Aus dem Institut für Veterinärbiochemie und Molekularbiologie
der Veterinärmedizinischen Fakultät
der Universität Zürich

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Optimised bacterial expression of three DNA quality control proteins, the checkpoint proteins Rad9, Rad1 and Hus1

INAUGURAL-DISSERTATION

zur Erlangung der Doktorwürde der
Veterinärmedizinischen Fakultät
der Universität Zürich

vorgelegt von

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Zürich 2003

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0. Abbreviations and explanations

A_{600}	Optical density, measured at 600nm wavelength
arg, ile, leu, pro	Amino-acids: the three letter code is used
ATP	Adenosine-triphosphate
AvaI, BamHI, EcoRV	DNA restriction enzymes
BSA	Bovine serum albumine
cDNA	Complementary DNA: single-stranded DNA complementary to a RNA
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL Detection System	A non-isotopic method for detecting protein immobilised on membranes
E.coli	*Escherichia coli*
EDTA	Ehtylendiamintetraacetate
FPLC	Fast protein, peptide and polynucleotide liquide chromatography
GST	Glutathione-S-transferase
GSTrap	Prepacked Glutathione Sepharose columns for one-step purification of GST-fusion proteins or other Glutathione binding proteins
HDAC1	Member of the histone deacetylase family
$(His)_6$-protein	6xHis-tagged protein
HiTrap Q	Anion exchange column, Q Sepharose High Performance
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani Medium: liquid bacterial medium consisting of bacto-tryptone, bacto-yeast extract and NaCl
LMW	Low molecular weight markers

MBP	Maltose-binding protein
NP-40	Nonidet P 40
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
pGEX-4T-3, pREP4, pMalc-2E, pRSET B, pET 24a+	Different bacterial expression vectors for heterologues protein production
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
Rad17, Rad9, Rad1 and Hus1	DNA quality control proteins, checkpoint proteins
RF-C	Replication factor C
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S. pombe	*Schizosaccharomyces pombe*
TBS	Tris-buffered saline
TBST	Tris buffered saline with additional Tween20
tRNA	Transfer Ribonucleic acid

Units:

hr/hrs	Hour/hours
kDa	Molecular mass; 1000 Da = 1 kDa
psi	Pounds per square inch
RCF	Relative centrifugal force, also referred to as g-force. It is relative to the earth's gravitational force and is identified as xg (times gravity) or RCF
rpm	Rotor speed in revolutions per minute
v/v	Volume per volume; percentage concentration in ml per 100ml fluid

1. Introduction and Problem

The DNA of mammals is constantly under pressure of the environment, thus leading to alteration in the DNA double helix. A mammalian cell has about 10,000 damaging events per day. On the other hand, DNA has to be very stable to maintain the genetic information from generation to generation. It is therefore not surprising that nature has developed many mechanisms to circumvent these problems. They include direct DNA repair, base excision repair and nucleotide excision repair. Moreover, cell cycle checkpoint control guarantees that cells entering or leaving DNA replication do not duplicate damaged DNA thus leading to mutations that might end up in alterations of the cellular phenotype (e.g. to cancer).

Rad9, Rad1 and Hus1 are three human proteins that play a critical role in cell cycle checkpoint signalling pathways. These checkpoints are activated after DNA damage and allow the slowing down of cell cycle progression and coordinate DNA repair. In recent studies it has been shown that Rad9, Rad1 and Hus1 interact in a head-to-tail manner (Burtelow *et al.*, 2001). Supported by molecular modelling studies these observations suggest a model in which Rad9, Rad1 and Hus1 form a stable, heterotrimeric ring similar to PCNA. This clamp could be loaded onto DNA by a clamp loader that recognizes DNA damage. Once loaded, this complex could be a platform for signalling and repair molecules (Dahm and Hubscher, 2002; Cai *et al.*, 2000; Caspari *et al.*, 2000; Venclovas and Thelen, 2000) (Figure 1).

These proteins are however difficult to express in bacteria. For expression of recombinant protein *E.coli* is normally the first choice, because it is quick, cheap and provides high yields of expression. Some eukaryotic proteins however do not fold properly in bacteria and form insoluble aggregates. Other problems working with bacteria include cell toxicity, inefficient translation, improper processing or post-translational modifications. Some of these problems can often be solved by testing different tagged proteins and *E.coli* strains, optimising expression conditions by lower growth temperature and varying the IPTG-concentration, cell density (A_{600}) and induction period. In this thesis, the bacterial expression of the three human checkpoint proteins Rad9, Rad1 and Hus1 was

successfully optimised by using different *E.coli* strains and testing several fusion proteins at different expression conditions.

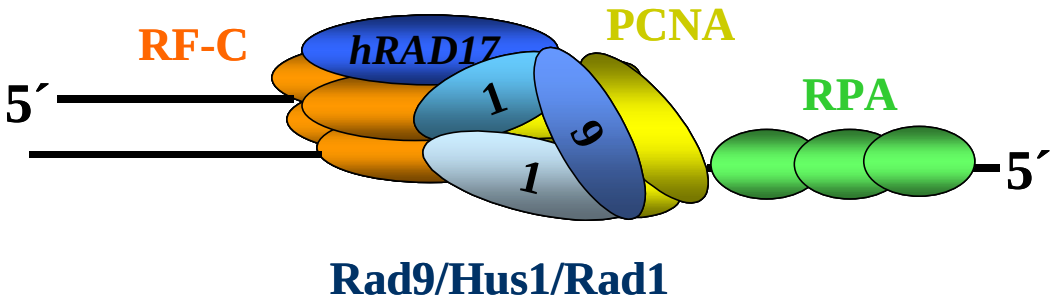

Figure 1. PCNA-like trimer consisting of Hus1, Rad1 and Rad9. It has been shown that Rad9, Rad1 and Hus1 interact in a head-to-tail manner and molecular modelling studies support a model in which Rad9, Rad1 and Hus1 form a stable, heterotrimeric ring similar to PCNA. Previously published data suggests a model, in which a Rad17/RF-C complex functions as a sensor or signal transducer. It binds to the Rad9/Hus1/Rad1 (9-1-1) complex and enables it to interact with PCNA. PCNA could provide communication between replication checkpoint control, DNA repair and the resumption of DNA replication (from Dahm and Hübscher, 2002).

1.1. Function of the three checkpoint proteins Rad9, Rad1 and Hus1

DNA damage provokes multiple cellular responses including the slowing down of cell cycle progression. The eukaryotic cell cycle consists of a number of tightly regulated events to ensure that DNA replication and cell division occur with high fidelity. In the presence of damaged or incompletely replicated DNA, the DNA damage (G2) and DNA replication (S-phase) checkpoints arrest eukaryotic cells at the G2/M transition (al-Khodairy *et al.*, 1994; al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Enoch and Nurse, 1990; Weinert and Hartwell, 1990; Weinert and Hartwell, 1988), thus providing time to repair damage or complete replication before the cell enters into mitosis. In *S. pombe* six checkpoint genes have been identified called Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1 (al-Khodairy *et al.*, 1994; al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). They are required for both G2 and S-phase checkpoint controls. Checkpoint rad-mutants are unable to block mitotic entry in response to DNA-

damaging agents or transient inhibition of DNA synthesis (al-Khodairy *et al.*, 1994; al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). Mitotic entry is regulated by dephosphorylation of the tyrosin-15 residue of the cyclin-dependent kinase Cdc2 (O'Connell *et al.*, 1997; Rhind *et al.*, 1997; Enoch and Nurse, 1990) (Figure 2). Phosphorylation/dephosphorylation of this residue is controlled primarily by the Cdc25 phosphatase and the Wee1 protein kinase, respectively. The activity of these two enzymes is regulated in turn by the kinases Chk1 (for Cdc25) and Cds1 (for Wee1) (Furnari *et al.*, 1997; Walworth *et al.*, 1993). Chk1 is part of the G2 checkpoint and is activated after DNA damage. It phosphorylates Cdc25, which thereby prevents Cdc2-dephosphorylation and mitotic entry (Furnari *et al.*, 1997; Walworth *et al.*, 1993). The S-phase checkpoint, on the other hand, is controlled by the activation of Cds1. It leads to phosphorylation of Wee1, which is able to phosphorylate Cdc2 and inhibit mitotic entry (Boddy *et al.*, 1998). The checkpoint rad proteins are placed upstream of the Cdc2 regulators in the signal transduction pathway because the checkpoint induced phosphorylation of Chk1 and Cds1 is dependent on the presence of all the checkpoint rad proteins (Lindsay *et al.*, 1998; Walworth and Bernards, 1996).

Many of the genes involved in G2 checkpoint pathways are conserved in the eukaryotic kingdom between yeast and humans (Table 1), suggesting that yeast G2 checkpoint signalling mechanisms may be similar to that of humans (Kostrub *et al.*, 1998; Parker *et al.*, 1998; Udell *et al.*, 1998; Cimprich *et al.*, 1996; Lieberman *et al.*, 1996).

Table 1. G2 checkpoint proteins in two yeasts and human

S. pombe	***S. cerevisiae***	***Homo sapiens***
Rad1	RAD17	hRad1
Rad3	MEC1	ATR/FRP1
Rad9	DDC1	hRad9
Rad17	RAD24	hRad17
Rad26	?	?
Hus1	?	hHus1
Chk1	CHK1	Chk1
Cds1	RAD53	Chk2

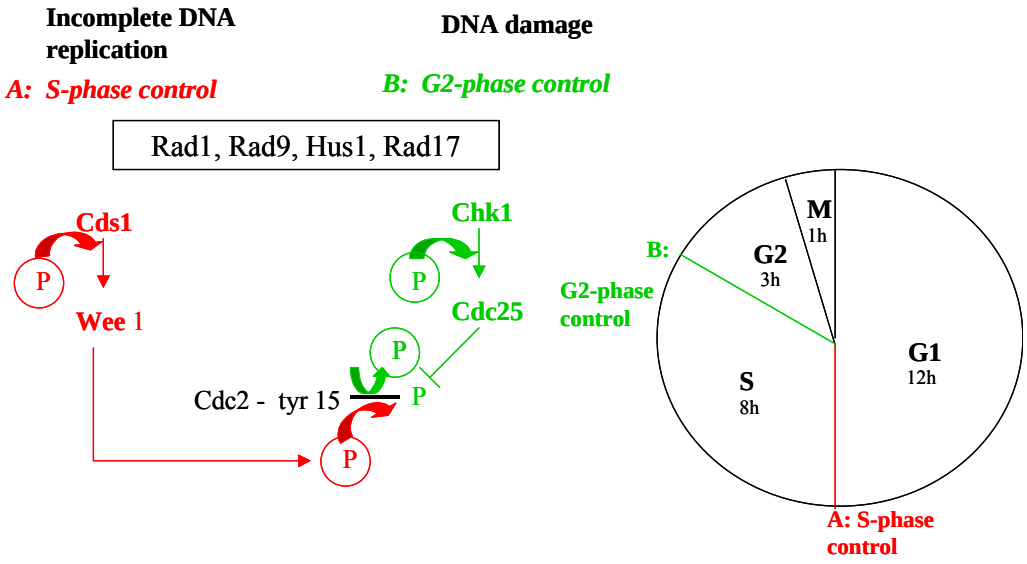

Figure 2. DNA damage (G2) and DNA replication (S-Phase) checkpoint control. Schematic representation of DNA damage (G2) and DNA replication (S-Phase) checkpoint, which arrest eukaryotic cells at the G2/M transition in the presence of damaged or incompletely replicated DNA to provide time for repair before the cell enters into mitosis. The checkpoint rad proteins are placed upstream of the Cdc2 regulators Chk1 and Cds1. The time declaration relates to mammalian cells in cell culture. For details see text.

Molecular modelling studies were used to propose functions for Rad9, Rad1, Hus1 and Rad17 (Cai *et al.*, 2000; Caspari *et al.*, 2000; Venclovas and Thelen, 2000). They support the model that Rad9, Rad1 and Hus1 form a heterotrimeric, PCNA-like clamp. PCNA is a homotrimeric ring that is loaded onto DNA by a clamp loader, replication factor C (RF-C) in an ATP-dependent manner (Hubscher *et al.*, 2002; Mossi and Hubscher, 1998). Once loaded, PCNA encircles the DNA as a sliding clamp and tethers replication proteins, including DNA polymerase δ. Rad9, Rad1 and Hus1 have been shown to interact by immunoprecipitation and by yeast-two-hybrid analysis (Caspari *et al.*, 2000; Hang and Lieberman, 2000; St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Following DNA damage, these three proteins were converted to an extraction-resistant, chromatin bound complex. By size-exclusion chromatography it has been shown that they interact in a head-to-tail manner, supporting the model of a circular organisation of these three proteins. Additionally, the *S. cerevisiae* homologue of Rad17 has been shown to interact with the four small subunits of RF-C in a stable complex (Green *et al.*, 2000).

Furthermore, Rad17 interacts with Rad9, Rad1 and Hus1 in a manner that requires an intact Rad17-ATP-binding domain (Rauen *et al.*, 2000).

All these data supports a model in which the four small subunits of RF-C form a complex with Rad17, which could act as a clamp-loader that recognizes DNA damage (Figure 1). Conformational changes in the subunits of the Rad17/RF-C complex associated with ATP-binding/hydrolysis could break the heterotrimeric ring and load it onto DNA. Once loaded, this Rad9-Rad1-Hus1-complex could be a platform to allow other enzymes to repair DNA lesions in a processive manner. The exact structure of the Rad9-Rad1-Hus1 interaction can only be confirmed by structural analysis of the purified complex. Additionally, each of these three checkpoint proteins could have an additional cellular role independent of this complex formation.

1.2. Expression of recombinant proteins in *E.coli*: general problems

Among the many systems available for heterologous protein production, the gram-negative bacterium *E.coli* remains one of the most attractive, firstly because of its ability to grow rapidly and at high density on inexpensive substrates, secondly due to its well characterized genetics and thirdly because of the large number of cloning vectors and mutant host strains available. With careful choice of host strains, vectors and growth conditions, most recombinant proteins can be cloned and expressed at high levels. However, mainly eukaryotic gene products can accumulate as insoluble aggregates in *E.coli*, so called inclusion bodies that lack functional activity. Other problems may include cell toxicity, inefficient translation, improper processing or posttranslational modifications.

1.2.1. Solubility problems

For biochemical and structural studies, it is important to obtain soluble and functionally active proteins, whereas for antibody-production, the antigen protein can be expressed either in native or denatured form. The conditions for optimal expression of individual proteins must be determined empirically. Optimal growth and expression conditions should be established with small-scale culture before large-scale protein purification is attempted in order to judge the effects of various growth conditions on expression levels

and solubility of recombinant proteins. The intracellular protein content is often a balance between the amount of soluble protein in the cells, the formation of inclusion bodies and protein degradation. Therefore, a time-course analysis of the expression level is useful to check the soluble and insoluble fractions at various times after induction and to establish the optimal induction period. By monitoring the cell growth before and especially after induction, the toxicity of an expressed protein can also be judged.

A reduction of the growth temperature following induction may be helpful to get higher levels of a soluble protein. Growth temperature affects both expression level and protein solubility and lower temperatures will reduce expression levels leading to a higher amount of soluble protein. By reducing the IPTG-concentration, the expression level can also be kept low. Alternatively, the culture can be induced at a higher cell density (optical density/ A_{600}) and the expression period can be reduced to a minimum. Furthermore, it may be reasonable to test different *E.coli* strains with different genetic background, because certain strains tolerate some proteins better than others and allow higher levels of expression before forming inclusion bodies.

Expression of a target protein as a fusion protein with a highly soluble partner can greatly improve its solubility. Fusion proteins were originally constructed to facilitate protein purification and immobilisation, but soon it became apparent that certain fusion partners could improve the solubility of passenger proteins. Different systems suitable for the construction of fusion proteins have been described, for example maltose-binding protein (MBP), thioredoxin and glutathione-S-transferase (GST). A likely reason for the improved folding (and/ or reduced degradation) of recombinant proteins is that the fusion partner rapidly reaches a native conformation and promotes the acquisition of correct structure in downstream folding units by favouring one-pathway isomerisation reactions. In a systematic comparison of the effectiveness of various fusion partners in increasing the solubility of six passenger polypeptides, it was found that MBP was far superior to either thioredoxin or glutathione-S-transferase as a ‘solubilizing’ partner (Kapust and Waugh, 1999).

1.2.2. Translation issue problems

The frequency of the codon-usage in eukaryotic cells and *E.coli* is different (Table 2). The problematic codons for bacterial expression of coding sequences derived from GC-rich genomes are arg codons (AGG or AGA) and pro codon (CCC). If coding sequences from organisms with AT-rich genomes are used for expression in *E.coli*, tRNAs recognising AGA or AGG (arg), ATA (ile) and CTA (leu) can be limiting. This can cause low or lack of protein synthesis, early termination and misincorporation of amino acids in the expressed protein (Forman *et al.*, 1998; Zahn, 1996). This so-called codon bias can be a significant obstacle for efficient expression of heterologous genes in *E.coli*. To solve this problem, new *E.coli* strains were developed, called *E.coli BL21-Codon Plus* strains. These strains derive from *E.coli-BL21* strains that lack the Lon protease and the OmpT protease. *E.coli BL21-Codon Plus-RIL* cells (*R*= arg, *I*= ile, *L*= leu) contain extra copies of the argU, ileY, and leuW tRNA genes. These genes encode tRNAs that recognize the arg codons AGA and AGG, the ile codon ATA, and the leu codon CTA. *E.coli BL21-*

Table 2. Codon preferences of selected organisms [1]

Organism	AGG arg	AGA arg	CGA arg	CTA leu	ATA ile	CCC pro
E.coli	1.4	2.1	3.1	3.2	4.1	4.3
Homo sapiens	11	11.3	6.1	6.5	6.9	**20.3**
Drosophila melanogaster	4.7	5.7	7.6	7.2	8.3	**18.6**
Caenorhabditis elegans	3.8	**15.6**	11.5	7.9	9.8	4.3
S. cerevisiae	9.3	**21.3**	3	13.4	**17.8**	6.8
Plasmodium falciparum	4.1	**20.2**	0.5	**15.2**	**33.2**	8.5
Clostridium pasteurianum	2.4	**32.8**	0.8	6	**52.5**	1
Pyrococcus honkoshii	**30.3**	**20.4**	1	**18**	**44.9**	10.1
Thermus aquaticus	13.7	1.4	1.4	3.2	2	**43**
Arabidopsis thaliana	10.9	**18.4**	6	9.8	12.6	5.2

1 Codon frequencies are expressed as codons used per 1,000 codons encountered. Codon frequencies of more than 15 codons/ 1,000 codons are shown in bold to help identify a codon bias that may cause problems for high-level expression in *E.coli*. The arg codons AGG and AGA are recognized by the same tRNA (product of the *arg*U gene) and should, therefore, be combined. However, regardless of the origin of the coding region of interest, each gene should be assessed individually. Even *E.coli* contain genes with poor codon usage for efficient translation (from Stratagene).

Codon Plus-RP cells (*R*= arg, *P*= pro) contain extra copies of the argU and proL genes, which encode tRNAs that recognize the arg codons AGA and AGG and the pro codon CCC. For that reason it can be reasonable to check the codon usage of the recombinant protein and to choose an appropriate *E.coli BL21-Codon Plus* strain.

1.3. Bacterial expression of the three recombinant checkpoint proteins Rad9, Rad1 and Hus1

It is known that the three checkpoint proteins Rad9, Rad1 and Hus1 are difficult to express in *E.coli*. Only little and incomplete data on bacterial expression of these proteins was found in the literature (Table 3). Eukaryotic expression systems can be used to produce soluble and functionally active protein for biochemical studies, but they are more complicated to handle than bacterial expression systems, more time consuming and yield relatively low protein amounts. Because bacterial systems are much easier to handle and provide higher amounts of protein, it was reasonable to try to establish optimal conditions to express each protein at reasonable levels, soluble and in a functionally active form. As mentioned above (see Introduction and Problem), different steps can be varied to improve bacterial expression and increase the amount of the soluble fraction. In this thesis, several *E.coli* strains were tested focussing on *E.coli BL21-Codon Plus* strains. Different tagged proteins were expressed and optimal conditions established in small-scale culture using a time-course analysis of the expressed protein. With optimised conditions, a large-scale protein expression was performed to purify high yields of soluble protein. By using these parameters, it was possible to express the two proteins Rad9 and Rad1 in good yields, and to some extend also Hus1. They could be purified to apparent homogeneity and were most likely properly folded since they could interact with each other in a so called “Dot blot Far Western” analysis.

Table 3. Bacterial expression of the three human checkpoint proteins Rad9, Rad1 and Hus1

PROTEIN	VECTOR	BACTERIAL STRAIN	EXPRESSION CONDITIONS	INDUCTION	RESULT	REFERENCES
Rad9						
GST-Rad9	pGEX-KG	not described	not described	not described	purified protein used to immunize rabbits	(Volkmer and Karnitz, 1999)
Rad9						
(His)6-Rad9	pRSET	Bacterial expression in	not described	not described	both forms:	(Bessho and Sancar,
MBP-Rad9	pMalc-2	*E.coli*			overproduced at reasonable levels, soluble, nuclease activity	2000)
Rad9						
GST-Rad9	pGEX1	Bacterial expression in *E.coli*	not described	not described	purified protein used to generate polyclonal chicken antibodies	(St Onge *et al.*, 1999)
Rad9						
GST-Rad9	pGEX-5X-3	Bacterial expression in	not described	not described	purified protein used for	(Chen *et al.*, 2001)
GST-Rad9(224-391)	pGEX-4T-1	*E.coli*			immunization	
Rad9					purified protein used for a peptide	(Schwartz *et al.*, 2002)
GST-Rad9	pGEX-4T-3	Bacterial culture	not described	not described	binding assay	
Rad1A		Bacterial expression in		0.5 mM IPTG	20% of Rad1A soluble	(Parker *et al.*, 1998)
(His)6-Rad1A	pRSET B	*E.coli BL21 (DE3)*	A_{600} = 0.5	20 hrs		
Rad1					purified protein used to immunize	(Volkmer and Karnitz,
(His)6-Rad1	pET 24a+	not described	not described	not described	rabbits	1999)
Hus1					*in vitro* interaction between	(Cai *et al.*, 2000)
GST-Hus1	pGEX-2TK	not described	not described	not described	GSTHus1 and HDAC1	
Hus1					purified protein used to immunize	(Volkmer and Karnitz,
(His)6-Hus1	pET 24a+	not described	not described	not described	rabbits	1999)

2. Materials and Methods

2.1. Recombinant Rad9, Rad1 and Hus1 proteins

The plasmid pGEX-4T-3-Rad1 was a gift from R. Freire (Teneriffa, Spain), pGEX-4T-3-Rad9 and pREP4-Hus1 were a gift from K. Dahm (this institute). The Hus1 cDNA was amplified from the plasmid by PCR using the primers 5`-CAG GAT CCC ATA TGA AGT TTC GG and 5`-TAC GAT ATC CTA GGA CAG CGC AGG. The PCR product was digested with BamHI and EcoRV and inserted into pGEX-4T-3 (Amersham Pharmacia Biotech) to obtain the expression vector pGEX-4T-3-Hus1. The pGEX-4T-3-Rad9 plasmid was digested with BamHI and AvaI and the fragment carrying the Rad9 coding sequence inserted into pMalc-2E (New England Biolabs) to obtain pMalc-2E-Rad9 plasmid allowing the expression of a maltose-binding protein (MBP)-Rad9 fusion protein. The insert sequence of all constructs was examined by partial sequencing.

2.2. Bacterial strains

E.coli-XL1-blue strain (Stratagene, La Jolla, CA 92037) was used as a host for plasmid amplification. *E.coli BL21-Codon Plus-RIL* and *E.coli BL21-Codon Plus-RP* strains (Stratagene, La Jolla, CA 92037) and *E.coli-TB1* (New England Biolabs) and *E.coli-TG1* (Stratagene, La Jolla, CA 92037) strains were used for expression of GSTRad1, GSTRad9, MBPRad9 and GSTHus1, respectively.

2.3. Buffer solutions

The following buffers were used: lysis buffer A: 1x PBS (pH 7.3), 1%(v/v) Triton X-100, 1mM EDTA, 1mM PMSF, 1mM DTT; lysis buffer B: 20mM Tris (pH 7.4), 200mM NaCl, 1mM PMSF, 1mM DTT; elution buffer A: 50mM Tris (pH 8), 150mM NaCl, 1mM EDTA, 1mM PMSF, 1mM DTT, 10mM Glutathione; elution buffer B: 20mM Tris (pH 7.4), 200mM NaCl, 1mM EDTA, 1mM PMSF, 1mM DTT, 10mM maltose; buffer A: 20mM Tris (pH 8), 0.01%(v/v) NP-40, 10%(v/v) glycerol, 75mM NaCl, 1mM PMSF, 1mM DTT; buffer B: 20mM Tris (pH 8), 0.01%(v/v) NP-40, 10%(v/v) glycerol, 1M

NaCl, 1mM PMSF, 1mM DTT; storage buffer: 50mM Tris (pH 7.4), 150mM NaCl, 1mM PMSF, 1mM EDTA, 1mM DTT, 10%(v/v) glycerol. SDS loading buffer: 50mM Tris (pH 6.8), 100mM DTT, 2%(v/v) SDS, 0.1%(v/v) bromphenol blue, 10%(v/v) glycerol.

2.4. Small-scale cultures of GSTRad1, GSTRad9, MBPRad9 and GSTHus1

The respective plasmids were transformed into *E.coli BL21-Codon Plus-RIL*, *E.coli BL21-Codon Plus-RP*, *E.coli-TB1* and *E.coli-TG1* according to the manufacturer's instructions. A single colony was inoculated in 100ml of LB medium (containing 10g/l NaCl, 10g/l bacto-tryptone, 5g/l bacto yeast extract, brought to pH 7 with 5M NaOH and supplemented with 100µg/ml ampicillin). For *E.coli BL21-Codon Plus* strains, 50µg/ml chlormaphenicol was added additionally. The culture was incubated at 250 rpm at 30°C overnight. 5ml of the overnight culture was added to six Erlenmeyer flasks containing 45ml of LB medium (supplemented with 100µg/ml ampicillin) and incubated to A_{600}= 0.5 or A_{600} = 1.0. For MBP-fusion proteins, LB medium was replaced by rich medium (containing 10g/l bacto-tryptone, 5g/l bacto yeast extract, 5g/l NaCl, 2g/l glucose, supplemented with 100µg/ml ampicillin). 1ml aliquots were collected from every flask (= uninduced cells), centrifuged at 6,500 rpm for 5 min. at 4°C and pellets were frozen at –20°C. High level expression was induced by the addition of IPTG to a final concentration of 0.1mM, 0.5mM and 1.0mM. The six small-scale cultures were incubated at 250 rpm at 27°C. Every hour A_{600} was measured and two separate 1ml fractions were collected from each culture. The samples were centrifuged at 6,500 rpm for 5 min. at 4°C and the pellets were frozen at –20°C and used for solubility-checks and protein-minipreparation experiments.

2.5. Solubility-check of GSTRad1, GSTRad9, MBPRad9 and GSTHus1

The pellets of the 1ml aliquots from small-scale cultures were resuspended in 200µl lysis buffer A for GST-tagged proteins and lysis buffer B for MBP-tagged proteins. The cells were lysed four times by freezing in liquid N_2 and thawing at 37°C. Lysates were separated by centrifugation at 13,000 rpm for 10 min. at 4°C. Supernatant (soluble

fraction) were transferred to new tubes and SDS loading buffer was added to 1x final concentration. The pellet (insoluble fraction) was resuspended in 100µl lysis buffer A for GST-tagged proteins and lysis buffer B for MBP-tagged proteins and SDS loading buffer was added to 1x final concentration. After boiling for 5 min. at 100°C, the soluble and insoluble fractions were analysed separately by SDS-PAGE followed by Coomassie staining.

The soluble fraction was analysed additionally by Western blot analysis. Therefore, the fractions were electrophoresed through a polyacrylamide gel. Proteins were electroblotted onto a nitrocellulose membrane and visualised by PonceauS staining to mark a low molecular weight marker LMW (Amersham Pharmacia Biotech). The membrane was destained using TBST (0.05%(v/v) Tween 20) and blocked for 1 hr in blocking solution (containing 5%(v/v) powder milk in TBST 0.05%(v/v) Tween20) at room temperature. α-GST or α-MBP polyclonal rabbit antibody were diluted 1:5,000 in blocking solution and the nitrocellulose membrane was incubated with diluted antibody for 1 hr at room temperature. Then the solution was removed and the membrane was washed two times for 20 min. and once for 10 min. with TBST (0.05%(v/v) Tween 20) and incubated again for 5 min. with blocking solution. HRP-conjugated goat anti-rabbit secondary antibody, diluted 1:10,000 in blocking solution, was added to the membrane and the membrane was incubated for 1 hr at room temperature, followed by 30 min. and 10 min. washing with TBST and 10 min. with TBS. Antibodies bound to protein antigens were detected by chemiluminescence using the ECL Detection System (Amersham Pharmacia Biotech) followed by exposure to an X-ray film (Eastman Kodak, Rochester, NY).

2.6. Protein-minipreparation

2.6.1. Protein-minipreparation of GST-tagged protein

The pellets of the 1 ml aliquots collected from the small-scale cultures were resuspended in lysis buffer A, lysed by repeated freezing and thawing in liquid N_2 and separated into soluble (supernatants) and insoluble protein fractions (pellets) by centrifugation as described for the solubility check (see 2.5.). Glutathione Sepharose 4B (Amersham

Pharmacia Biotech) was washed with 1xPBS and equilibrated with lysis buffer A resulting in a 50% slurry. 20µl slurry was then added to each supernatant and rotated for 1 hr at 4°C. The suspension was centrifuged at 500g for 5 min. and the supernatant removed (unbound protein). Glutathione Sepharose 4B beads were washed 3 times with 500µl lysis buffer A and once with 500µl lysis buffer A without Triton X-100. The pellet was finally resuspended in 50µl 1x SDS loading buffer and boiled for 5 min. at 100°C, followed by centrifugation at 6,500 rpm for 1 min.. 25µl of the samples containing protein eluted from the beads were resolved by SDS-PAGE and stained by Coomassie, 25µl of samples were analysed by immunoblot analysis using the same procedure as described for the solubility check.

2.6.2. Protein-minipreparation of MBP-tagged protein

For the MBP-tagged protein, the same methods were used as described for GST-tagged protein, with the following alterations: the pellets of the 1 ml aliquots from the small-scale cultures were resuspended in lysis buffer B. Amylose–resin (New England Biolabs) was washed and equilibrated with lysis buffer B resulting in a 50% slurry. 50µl slurry was added to each supernatant and rotated for 1 hr at 4°C. The Amylose-resin was washed only once with 1ml lysis buffer B.

2.7. Purification of GSTRad1, MBPRad9 and GSTHus1

2.7.1. MBPRad9

2.7.1.1. Expression of a 1.6 l culture

1µg of the plasmid pMalc-2E-Rad9 was transformed into *E.coli BL21-Codon Plus-RP* cells following the manufacturer's instructions. 100µl of transformed cells were inoculated in 400 ml LB medium (supplemented with 50µg/ml ampicillin and 50µg/ml chloramphenicol). Then the culture was incubated at 250 rpm at 30°C overnight. 180 ml of the overnight culture was added to two Erlenmeyer flasks containing 700ml of rich medium, containing 10g/l bacto-tryptone, 5g/l bacto yeast extract, 5g/l NaCl, 2g/l

glucose, supplemented with 100µg/ml ampicillin, and incubated to A_{600}=1.0. A 1ml aliquot was collected, centrifuged and the pellet was frozen at –20°C (= uninduced cells). IPTG was then added to a final concentration of 0.5mM and the culture incubated at 250 rpm for 3 hrs at 27°C. A 1ml aliquot was collected, centrifuged and frozen at –20°C (= induced cells). The cells were harvested by centrifugation at 7,000 rpm for 30 min. at 4°C, the pellet resuspended in 1xPBS and transferred to 50ml Falcon-Tubes. After centrifugation at 4,000 rpm for 30 min. at 4°, the pellet was frozen at –80°C until further use.

2.7.1.2. Purification of MBPRad9

2.7.1.2.1. Amylose-resin, batch-purification

The bacterial pellet was resuspended in 40ml lysis buffer B and the bacteria were lysed two times by passage through a French press at 1200 psi. The lysate was separated into a soluble (supernatant) and an insoluble fraction (pellets) by centrifugation at 16,000 rpm for 30 min. at 4°C. The pellet was frozen at –20°C and a 1ml aliquot of the supernatant was collected, centrifuged and frozen at –20°C (load). 1ml amylose-resin was washed two times with 20ml lysis buffer B and equilibrated with 500µl lysis buffer, resulting in a 50% slurry. The soluble protein fraction was rotated with the amylose-resin slurry for 2 hrs at 4°C. After centrifugation at 500g for 5 min., the supernatant was removed (unbound protein) and the resin washed 3 times with 10ml, 5ml and 5ml lysis buffer B. The pooled wash fractions were saved. Bound protein was eluted 6 times with 500µl elution buffer B by rotating each for 10 min. at 4°C, centrifugation at 500g for 5 min. and collecting the supernatant. Load, unbound protein, wash and 10µl of each collected fraction of eluted protein were resolved by SDS-PAGE and analysed by Coomassie staining. Those fractions containing MBPRad9 were pooled and dialysed overnight at 4°C against 1 l of buffer A.

2.7.1.2.2. Anion exchanger HiTrap Q

A prepacked 1ml HiTrap Q anion exchange column (Amersham Pharmacia Biotech) was equilibrated with 10ml buffer A at a flow rate of 1ml/min. The pooled and dialysed fractions were loaded onto the column at a flow rate of 0.25ml/min using a FPLC-system. The flow through was saved. The column was washed with 2.5ml buffer A at a flow rate of 0.5ml/min. Bound protein was eluted with buffer B at a flow rate of 0.5ml/min with a continuous 10ml salt gradient at an increase in ionic strength from 75mM to 1M NaCl. Fractions of 0.25ml were collected and the load, flow through and 10µl of each fraction resolved by SDS-PAGE and analysed by Coomassie staining. Those fractions containing MBPRad9 were pooled to a main pool and two side pools and dialysed overnight at 4°C against 1 l storage buffer. The pools were aliquoted into 100µl and frozen at –80°C until further use. The protein content of each pool was quantified by Bradford assay and analysed by SDS-PAGE and Coomassie staining.

2.7.2. GSTRad1

2.7.2.1. Expression of a 2 l culture

1µg of the plasmid pGEX-4T-3-Rad1 was transformed into *E.coli BL21-Codon Plus-RIL* cells following the manufacturer's instructions. A single colony was inoculated in 300 ml LB medium (supplemented with 100µg/ml ampicillin and 50µg/ml chloramphenicol). The culture was incubated at 250 rpm at 30°C overnight. 70 ml of the overnight culture was added to three Erlenmeyer flasks containing 630ml of LB medium (supplemented with 100µg/ml ampicillin), and incubated to A_{600}=1.0. A 1ml aliquot was collected, centrifuged and the pellet was frozen at –20°C (= uninduced cells). IPTG was added to a final concentration of 1mM and the culture incubated at 250 rpm for 3 hrs at 25°C. A 1ml aliquot was collected, centrifuged and frozen at –20°C (= induced cells). The cells were harvested by centrifugation at 7,000 rpm for 30 min. at 4°C, the pellet was resuspended in 1xPBS and transferred to 50ml Falcon-Tubes. After centrifugation at 4,000 rpm for 30 min. at 4°C, the pellet was frozen at –80°C until further use.

2.7.2.2. Purification of GSTRad1

2.7.2.2.1. GSTrap

The bacterial pellet was resuspended in 40ml lysis buffer A and the bacteria were lysed two times by passage through a French press at 1200 psi. The lysate was separated into a soluble (supernatant) and an insoluble fraction (pellet) by centrifugation at 16,000 rpm for 30 min. at 4°C. The pellet was frozen at –20°C and a 1ml aliquot of the supernatant collected, centrifuged and frozen at –20°C (load). A prepacked 1ml GSTrap column (Amersham Pharmacia Biotech) was equilibrated with 10ml lysis buffer A. The supernatant was passed through a 45µm filter and applied to the column at a flow rate of 0.5 ml/min by using a FPLC-system. The flow through was saved and the column washed with 15ml lysis buffer A followed by 15ml lysis buffer A without Triton X-100 at a flow rate of 0.5ml/min..The pooled wash fractions were saved. The bound protein was eluted with 10ml elution buffer A at a flow rate of 0.5ml/min and fractions of 0.25 ml were collected. Load, flow through, wash and 10µl of each fraction were resolved by SDS-PAGE and analysed by Coomassie staining. Those fractions containing GSTRad1 were pooled and dialysed overnight at 4°C against 1 l of buffer A.

2.7.2.2.2. Anion exchanger HiTrap Q

A prepacked 1ml HiTrap Q anion exchange column (Amersham Pharmacia Biotech) was equilibrated with 10ml of buffer A at 1ml/min.. The pooled and dialysed fractions were loaded onto the column at a flow rate of 0.25ml/min by using a FPLC-system. The flow through was saved and the column was washed with 2.5ml buffer A at a flow rate of 0.5ml/min. Bound protein was eluted with buffer B at a flow rate of 0.5ml/min with a continuous 10ml salt gradient at an increase in ionic strength from 75mM to 1M NaCl. Fractions of 0.25ml were collected and the load, flow through and 10µl of each fraction resolved by SDS-PAGE and analysed by Coomassie staining. Those fractions containing GSTRad1 were pooled to a main pool and two side pools and dialysed overnight at 4°C against 1 l storage buffer. The pools were aliquoted into 100µl and frozen at –80°C until further use. The protein content of each pool was quantified by Bradford assay and analysed by SDS-PAGE and subsequent Coomassie staining or immunoblot analysis.

2.7.3. GSTHus1

2.7.3.1. Expression of a 4 l culture

1µg of the plasmid pGEX-4T-3-Hus1 was transformed into *E.coli -TB1* cells following the manufacturer's instructions. 100µl transformed cells were inoculated in 500 ml LB medium (supplemented with 50µg/ml ampicillin). The culture was incubated at 250 rpm at 30°C overnight. 100 ml of the overnight culture was added to four Erlenmeyer flasks containing 900ml of LB medium (supplemented with 50µg/ml ampicillin), and incubated to A_{600}= 0.4. A 1ml aliquot was collected, centrifuged and the pellet frozen at –20°C (= uninduced cells). IPTG was added to a final concentration of 0.5mM and the culture incubated at 250 rpm for 5 hrs at 27°C. A 1ml aliquot was collected, centrifuged and frozen at –20°C (= induced cells). The cells were harvested by centrifugation at 7,000 rpm for 30 min. at 4°C, the pellet resuspended in 1xPBS and transferred to 50ml Falcon-Tubes. After centrifugation at 4,000 rpm for 30 min. at 4°C, the pellet was frozen at -80°C until further use.

2.7.3.2. Purification of GSTHus1

For purification of GSTHus1, the same columns, methods and buffers were used as described for GSTRad1.

2.8. Dot blot Far Western analysis

Purified MBPRad9 (3µg, 5µg and 7µg) was dotted onto nitrocellulose (0.22µm Osmonics, Westborough) and air dried for 30 min. at 4°C. The nitrocellulose was then rehydrated 5 min. in aqua bidest and blocked in TBS, 5%(v/v) powder milk and 0.05%(v/v) Tween 20 for 1 hr at 4°C, followed by two short washes of 1 min. with TBS 0.05%(v/v) Tween 20, 0.1%(v/v) BSA. The membrane was then incubated with GSTRad1 or GSTHus1 (1µg/ml) in TBS supplemented with 0.05%(v/v) Tween 20, 0.1%(v/v) BSA, 1mM DTT and 1mM PMSF for 3 hrs at 4°C. The membrane was washed four times for 10 min. at 4°C in TBS containing 0.05%(v/v) Tween 20. The second wash

contained 0.0001%(v/v) glutaraldehyde. Then the nitrocellulose was again blocked in the above mentioned buffer for 1 hr at room temperature. An immunoblot analysis was then performed to detect the presence of GSTRad1 or GSTHus1 using α-GST polyclonal rabbit antibody (Santa Cruz Biotechnology, California) previously diluted 1:5,000 in blocking solution. The nitrocellulose membrane was incubated with diluted antibody for 1 hr at room temperature. Then the membrane was washed two times for 20 min. and 10 min. each with TBST (0.05%(v/v) Tween 20) and blocked again for 5 min.. Finally HRP-conjugated anti-rabbit secondary antibody, diluted 1:10,000 in blocking solution, was added, the membrane incubated for 1 hr at room temperature, followed by 30 min. and 10 min. washings with TBST (0.05%(v/v) Tween 20) and 10 min. with TBS. Antibodies bound to the two proteins were detected by chemiluminescence by using the ECL Detection System (Amersham Pharmacia Biotech) followed by exposure to an X-ray film (Eastman Kodak, Rochester, NY).

3. Results

3.1. Rad1

3.1.1. Fusion protein and expression conditions

There is only little and incomplete data published in the literature on bacterial expression of Rad1 (Table 3). One paper describes bacterial expression of $(His)_6$-tagged Rad1 using a pRSET B vector (Parker *et al.*, 1998). Two truncated forms of Rad1, called Rad1A and Rad1B were expressed in *E.coli BL21 (DE3)*. Approximately 20% of expressed Rad1A was found in the soluble fraction, whereas for Rad1B, the expressed recombinant protein was almost completely insoluble. A second paper described bacterial expression of Rad1 using a pET 24a+ vector (Volkmer and Karnitz, 1999). But they published no data about bacterial strain, expression conditions and solubility of the purified protein, which was used to immunize rabbits. Because of this incomplete information in the literature, it was reasonable to express Rad1 as a different fusion-protein, using a pGEX-4T-3 plasmid to get a GST-tagged protein, GSTRad1.

3.1.2. Bacterial strain

GSTRad1 is a 58.5 kDa protein. First, the codon sequence of this protein was checked, focussing on the critical codons arg (AGG, AGA, CGA), leu (CTA), ile (ATA) and pro (CCC) (Table 4).

Table 4. Codon frequencies of critical codons in GSTRad1 and *E.coli* [1]

Amino-acid	Codon	GSTRad1	*E.coli*
arg	AGG	**15.3**	1.4
	AGA	12.8	2.1
	CGA	12.8	3.1
leu	CTA	10.3	3.2
ile	ATA	**25.6**	4.1
pro	CCC	4.3	4.3

[1] Codon frequencies are expressed as codons used per 1,000 codons encountered. The two codon frequencies of GSTRad1 that differ most from *E.coli* are shown in bold to help identify a codon bias that may cause problems for high-level expression in *E.coli*.

It was found that the most critical codons are AGG (arg), followed by ATA (ile) and AGA (arg), less critical are CGA(arg), CTA (leu) and CCC (pro). Therefore, it was reasonable to express Rad1 in *E.coli BL21-Codon Plus-RIL* cells containing extra copies of the argU, ileY and leuW tRNA genes (see Introduction and Problem).

3.1.3. Small scale cultures and solubility check of GSTRad1

3.1.3.1. GSTRad1 expressed in *E.coli BL21-Codon Plus-RIL*

To establish optimal growth and expression conditions for GSTRad1 in *E.coli BL21-Codon Plus-RIL* cells, small scale cultures were used and six different expression conditions were tested (Table 5).

Table 5. Expression conditions and cell growth after induction of GSTRad1 in *E.coli -Codon Plus-RIL* cells

Expression conditions				1 h	2 h	3 h	4 h	5 h	6 h
A_{600}=1	25°C	1mM IPTG	LB	A_{600} 1.23	A_{600} 1.42	A_{600} 1.67	A_{600} 1.82	A_{600} 2.05	A_{600} 2.05
	25°C	0.5mM IPTG	LB	A_{600} 1.26	A_{600} 1.42	A_{600} 1.74	A_{600} 1.87	A_{600} 2.06	A_{600} 2.38
	25°C	0.1mM IPTG	LB	A_{600} 1.29	A_{600} 1.44	A_{600} 1.77	A_{600} 1.94	A_{600} 2.19	A_{600} 2.37
A_{600}=0.5	25°C	1mM IPTG	LB	A_{600} 0.75	A_{600} 0.88	A_{600} 1.17	A_{600} 1.32	A_{600} 1.43	A_{600} 1.70
	25°C	0.5mM IPTG	LB	A_{600} 0.78	A_{600} 0.92	A_{600} 1.16	A_{600} 1.34	A_{600} 1.46	A_{600} 1.64
	25°C	0.1mM IPTG	LB	A_{600} 0.77	A_{600} 0.95	A_{600} 1.22	A_{600} 1.37	A_{600} 1.60	A_{600} 1.77

Two separate 1ml fractions were collected from each culture before (uninduced cells) and every hour after induction (induced cells) to get a time course analysis of the expression level and to establish the optimal induction period. A_{600} was measured every hour to check the cell growth after induction and thereby judging the toxicity of the expressed GSTRad1 protein on the bacterial cells (Table 5). The cells of the collected fractions were lysed and lysates were separated by centrifugation. The soluble (supernatants) and insoluble fractions (pellets) were analysed separately by SDS-PAGE followed by Coomassie staining (Figure 3A). The soluble fractions were analysed additionally by immunoblot analysis (Figure 3B). According to this small-scale culture and solubility check, GSTRad1 is expressed, as expected, prevailing insoluble and can be found in increasing amount in the pellet over the expression period (Figure 3A, not all conditions shown). On the other hand a reasonable amount of soluble protein without distinct degradation was present in each condition tested (Figure 3B, not all conditions shown). The amount of soluble expressed GSTRad1 detected in the supernatant by immunoblot analysis (Figure 3B) stayed almost constant over the expression period and did not differ significantly between the different expression conditions tested. In addition, according to the measured optical density (A_{600}) (Table 5), it can be mentioned that GSTRad1 showed no obvious toxicity to the bacterial cells.

To test the correct folding of expressed GSTRad1 protein by its binding to Glutathione Sepharose 4B, each collected fraction was additionally analysed by protein-

minipreparation. Therefore, the soluble fraction of each expression condition was incubated with Glutathione Sepharose 4B and the bound protein fraction was analysed by SDS-PAGE followed by subsequent Coomassie staining (Figure 3C, not all conditions shown) or immunoblot analysis (Figure 3D, not all conditions shown). Soluble and bound GSTRad1 recombinant protein bacterially expressed in *E.coli BL21-Codon Plus-RIL* could be found in each expression condition tested, but the amount of bound protein was higher at A_{600}=1 than at A_{600}=0.5 (Figure 3C). It appeared that protein-minipreparation by Glutathione Sepharose 4B can lead to some protein degradation that could be detected by immunoblot analysis and that the amount of degraded protein was increasing over the expression period (Figure 3D, not all conditions shown).

3.1.4. Optimised expression conditions for Rad1

Rad1 seemed to be expressed at reasonable levels as a GST-tagged fusion protein in *E.coli BL21-Codon Plus-RIL*, and although the amount of insoluble expressed GSTRad1 recombinant protein was higher than the soluble protein fraction, soluble protein could be found in every condition tested showing no distinct degradation. The amount of soluble GSTRad1 protein that bound to Glutathione Sepharose 4B was significantly higher at A_{600}=1. Degradation was present at every expression condition tested, but it appeared to increase over the expression period. Therefore a high cell density (A_{600}) at the time of induction and a high concentration of IPTG was chosen to get a high level of expression during the induction period, but thereby keeping the expression period short to limit protein degradation. The expression condition chosen was A_{600}=1, 1mM IPTG, at 25°C for 3hrs.

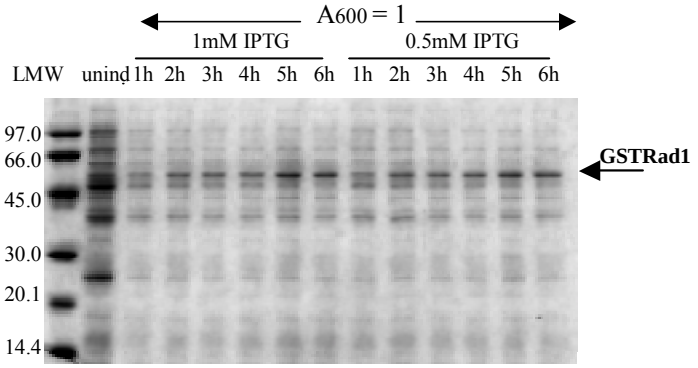

Figure 3A. Expression of GSTRad1 in *E.coli BL21-Codon Plus-RIL*, pellet, Coomassie stained gel. A Coomassie blue-stained 12% SDS-polyacrylamide gel of the insoluble fractions (pellets) of GSTRad1 expressed in *E.coli BL21-Codon Plus-RIL* strain is shown. 10µl of each collected 1ml fraction was loaded. Lanes: 1, low molecular weight markers; 2, uninduced cells, 3-8, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 9-14, culture induced at A600=1 with 0.5mM IPTG, 1-6 hrs of induction. The position of the GSTRad1 band is indicated by an arrow on the right.

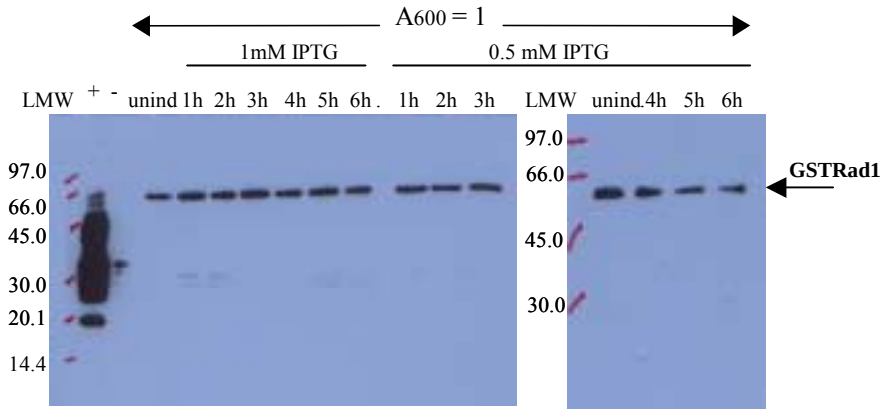

Figure 3B. Expression of GSTRad1 in *E.coli BL21-Codon Plus-RIL*, supernatant, immunoblot analysis. 25µl of the soluble fraction of each collected 1ml fraction was analysed by 12% SDS-polyacrylamide gel followed by Western blot analysis using a α-GST polyclonal rabbit antibody diluted 1: 5,000. *Left side:* Lanes: 1, low molecular weight markers; 2, positive control GSTRad17; 3, negative control Aldolase; 4, uninduced cells; 5-10, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 11-13, culture induced at A600=1 with 0.5mM IPTG, 1-3 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 4-6 hrs of induction. The position of the GSTRad1 band is indicated by an arrow on the right.

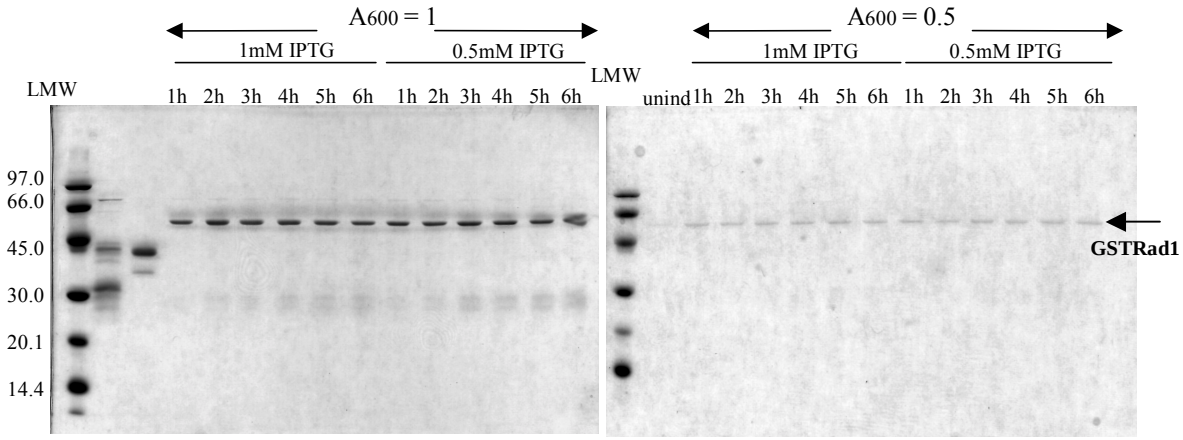

Figure 3C. Expression of GSTRad1 in *E.coli BL21-Codon Plus-RIL*, protein-minipreparation of the soluble fraction, Coomassie stained gel. The soluble fraction of each collected 1ml aliquot was incubated with washed and equilibrated Glutathione Sepharose 4B 50% slurry. Unbound protein was removed, Glutathione Sepharose 4B beads were washed and protein was eluted as described in Materials and Methods. 20µl of the samples containing eluted protein were resolved by SDS-PAGE and stained by Coomassie. *Left side:* Lanes: 1, low molecular weight markers; 2, GSTRad17; 3, Aldolase; 4-9, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 10-15, culture induced at A600=1 with 0.5mM IPTG, 1-6 hrs of induction; *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-8, culture induced at A600=0.5 with 1mM IPTG, 1-6 hrs of induction; 9-14, culture induced at A600=0.5 with 0.5mM IPTG, 1-6 hrs of induction. The position of the GSTRad1 band is indicated on the right.

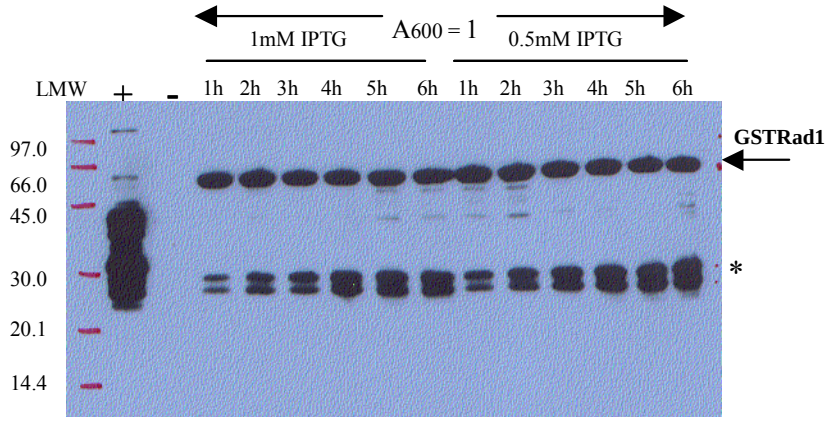

Figure 3D. **Expression of GSTRad1 in *E.coli BL21-Codon Plus-RIL*, protein-minipreparation of the soluble fraction, immunoblot analysis.** The fractions were treated as described for Figure 3C. 20µl of the samples containing eluted protein were resolved by SDS-PAGE and analysed by Western blot analysis using a α-GST polyclonal rabbit antibody. Lanes: 1, low molecular weight markers; 2, positive control GSTRad17; 3, negative control Aldolase; 4-9, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 10-15, culture induced at A600=1 with 0.5mM IPTG, 1-6 hrs of induction. The position of the GSTRad1 band is indicated by an arrow on the right. Degradation is indicated by * .

3.1.5. Big culture and purification of GSTRad1 in *E.coli BL21-Codon Plus-RIL*

3.1.5.1. Purification of GSTRad1 by a GSTrap column

pGEX-4T-3-Rad1 plasmid was transformed into *E.coli BL21-Codon Plus-RIL* cells as described above. A single transformed colony was inoculated in LB medium, which was then expanded to a 2 l culture. Expression was started at A_{600}=1 by adding IPTG to a final concentration of 1mM and culture was incubated at 25°C for 3 hrs at 250 rpm. The cells were harvested by centrifugation and resuspended in lysis buffer as described above. Bacteria were lysed by passage through a French press and separated into a soluble and insoluble fraction by centrifugation. The filtered soluble fraction was applied to a prepacked and equilibrated GSTrap column using a FPLC-system. The column was washed, bound protein was eluted with elution buffer and fractions were collected. Load, flow through, wash and the collected fractions were analysed by SDS-PAGE and Coomassie staining (Figure 4A). Soluble purified GSTRad1 protein was present in fractions 5-28. After this first purification step, GSTRad1 was not that concentrated and some degradation and contamination were present. Therefore a second purification step was necessary and for this the fractions containing GSTRad1 (fractions 5-28) were pooled.

3.1.5.2. Purification of GSTRad1 by an anion exchanger HiTrap Q

Based on the codon sequence of GSTRad1, its pI ($pI_{GSTRad1}$ = 5.03) was calculated and a anion exchanger (HiTrap Q) was chosen as next purification step. Enrichment of the target protein GSTRad1 is thereby achieved by choosing a start buffer (buffer A) with a pH at least 1 unit above the pI of substance to be bound ($pI_{GSTRad1}$= 5.03). Exposed to this buffer A, GSTRad1 carries a negative charge and binds to an anion exchanger (HiTrap Q). For buffer A pH 8 was chosen and the pooled fractions were dialysed against this buffer A and loaded onto the column using a FPLC-system. After several washing steps, bound protein was eluted with a continuous salt gradient at an increase in ionic strength. Fractions were collected and load, flow through, wash and the collected fractions were analysed by SDS-PAGE and Coomassie staining (Figure 4B). GSTRad1 was mostly present in fractions 13-28, especially in fractions 17-22. Some GSTRad1 recombinant

protein did not bind properly to the column and was therefore present in the flow through. So it was concluded that pH 8 of buffer A was probably not high enough for a sufficient binding of GSTRad1 to the anion exchanger. On the other hand, a higher chosen pH for buffer A could have led to additional protein degradation, what fortunately did not happen during this second purification step. Nevertheless, some degraded protein was still present and eluted exactly at the same fractions as non-degraded GSTRad1 protein. According to this observation, it was concluded that this degradation would be very hard to separate by further purification steps and probably additional degradation would appear, as observed during protein-minipreparation of GSTRad1 (see above). Therefore, no further purification step was done for recombinant GSTRad1 protein. The fractions containing GSTRad1 were pooled to a main pool (Pool 1, fractions 17-22) and two side pools (Pool 2, fractions 13-16; Pool 3, fractions 23-28). The protein content of each pool was quantified by Bradford assay (see Discussion, Table 10) and analysed by SDS-PAGE followed by subsequent Coomassie staining (Figure 4C) or immunoblot analysis (data not shown).The pools were finally dialysed against a storage buffer and frozen at –80°C until further use (see Activity test, Figure 12).

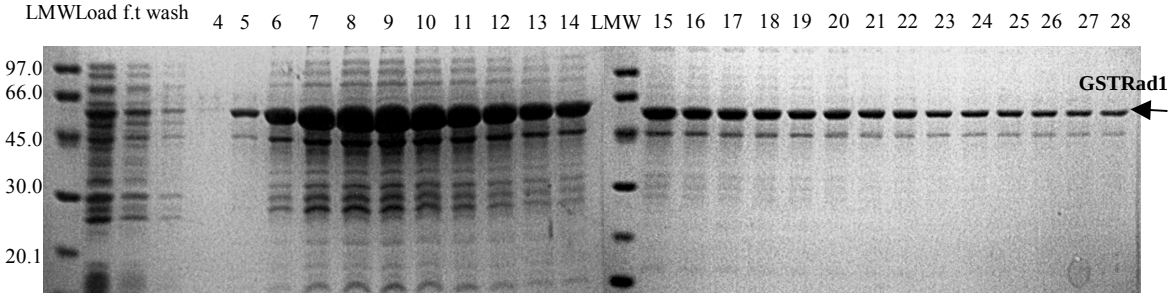

Figure 4A. GSTRad1 purification , 2 l culture, GSTrap column, Coomassie stained gel. The bacterial pellet of a 2 l culture of *E.coli BL21-Codon Plus-RIL* expressing GSTRad1 was resuspended, lysed and the soluble fraction filtered as described in Materials and Methods. The filtered supernatant was applied to an equilibrated GSTrap column using a FPLC-system, followed by several washing steps. Bound GSTRad1 protein was eluted from the column as described above. 0.25ml fractions were collected and 1µl load, 1µl flow through , 1µl wash and 10µl of each collected fraction were resolved by 12% SDS-PAGE and analysed by Coomassie staining. *Left side:* Lanes: 1, low molecular weight markers; 2, load; 3, flow through; 4, wash; 5-15, 10µl of each fraction 4-14 containing GSTRad1 protein eluted from the GSTrap column. *Right side:* Lanes: 1, low molecular weight markers; 2-15, 10µl of each fraction 15-28 containing GSTRad1 protein eluted from the GSTrap column. The position of the GSTRad1 band is indicated by an arrow on the right.

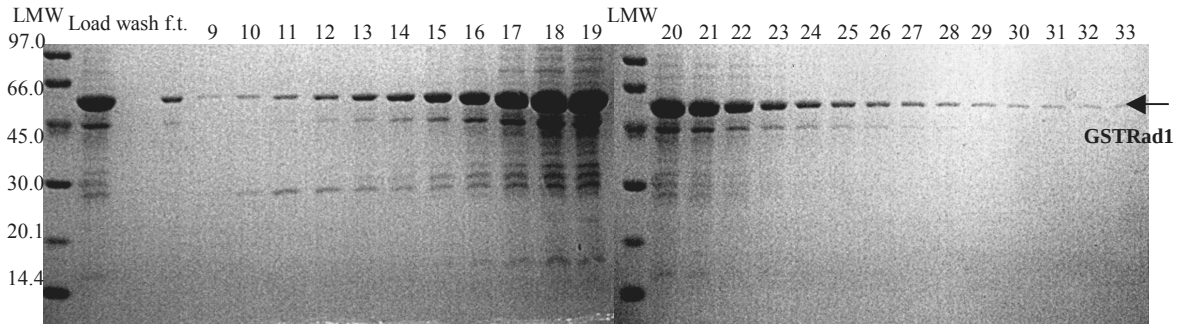

Figure 4B. GSTRad1 purification, collected fractions after anion exchange column, Coomassie stained gel. Pooled and dialysed fractions from 4A. were loaded onto a prepacked and equilibrated HiTrap Q anion exchange column as described in Materials and Methods. The column was washed, the protein eluted and 0.25ml fractions were collected. Aliquots of the load, flow through and 10µl of each collected 0.25ml fraction were analysed by 12% SDS-PAGE followed by Coomassie staining. *Left side:* Lanes: 1, low molecular weight markers; 2, 10µl load; 3, 10µl wash; 4, 10µl flow through; 5-15, 10µl of each fraction 9-19 containing GSTRad1 protein eluted from the anion exchange column. *Right side:* Lanes: 1, low molecular weight markers; 2-15, 10µl of each fraction 20-33 containing GSTRad1 protein eluted from the anion exchange column. The position of the GSTRad1 band is indicated by an arrow on the right.

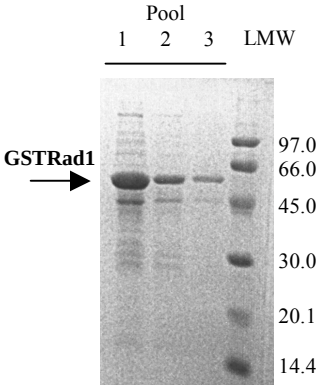

Figure 4C. GSTRad1 purification, Pool 1, Pool 2 and Pool 3 after two-step purification, Coomassie stained gel. After the anion exchange column HiTrap Q, fractions 17-22, containing the main part of GSTRad1 were pooled to a main pool, Pool 1, and analysed by SDS-PAGE followed by Coomassie staining. Two side pools, Pool 2 and Pool 3, containing the fractions 13-16 and 23-28, respectively, were additionally analysed. Lanes: 1, 10µl of Pool 1; 2, 10µl of Pool 2; 3, 10µl of Pool 3; 4, low molecular weight markers. The position of the GSTRad1 band is indicated by an arrow on the left.

3.2. Rad9

3.2.1. Fusion protein and expression conditions

Although a considerable amount of literature is published on structure, function and interaction of Rad9, only little data is available on its bacterial expression. Most of the authors prefer other expression systems, especially yeast cells, probably knowing about the problems of bacterial expression of this human checkpoint protein. Data published on bacterial expression of recombinant Rad9 protein is shown in Table 3. Four research groups tried to express recombinant Rad9 in a pGEX-vector to get a GST-tagged fusion protein, GSTRad9 (Schwartz *et al.*, 2002; Chen *et al.*, 2001; St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). All the authors except Schwartz et al. used purified GSTRad9 to immunize rabbits and to get anti-GSTRad9-antibody. Schwartz et al. used purified GSTRad9 recombinant protein for a functional assay, a peptide binding assay. Nevertheless, there is no data about the solubility of expressed GSTRad9 and its expression level. Secondly, a paper was found which describes bacterial expression of recombinant Rad9 (Bessho and Sancar, 2000). These authors used a pRSET and pMalc-2 expression vector to get $(His)_6$-Rad9 and MBPRad9, respectively, and expressed it in *E.coli.* They mentioned that both forms were overproduced at "reasonable" levels and were soluble. Nevertheless they described that the $(His)_6$-Rad9 form contained some contaminants even after four-column purification procedure so that they conducted most experiments with MBPRad9 recombinant protein. Even in this paper nothing is mentioned about exact *E.coli* strain or the expression conditions used. According to the published data (Schwartz *et al.*, 2002; Chen *et al.*, 2001; St Onge *et al.*, 1999; Volkmer and Karnitz, 1999) the bacterial expression of GSTRad9 was tested using a pGEX-4T-3 expression vector. Additionally, a pMalc-2E expression vector was used to get a MBP-fusion protein, MBPRad9 (Bessho and Sancar, 2000).

3.2.2. Bacterial strain

Based on the encouraging results with bacterial expression of GSTRad1, an *E.coli BL21-Codon Plus s*train was first tested to circumvent the problem of codon bias (see Introduction and Problem). Therefore the codon sequence of GSTRad9 and MBPRad9 was checked and codon frequencies were compared with *E.coli* (Table 6). GSTRad9 contains 616 codons and has a molecular weight (MW) of 68.7 kDa whereas MBPRad9 contains 783 codons and its moelcular weight is 85.4 kDa.

Table 6. Codon frequencies of critical codons in GSTRad9, MBPRad9 and *E.coli* [1]

Amino-acid	Codon	GSTRad9	MBPRad9	*E.coli*
arg	AGG	3.25	3.83	1.4
	AGA	3.25	0	2.1
	CGA	3.25	0	3.1
leu	CTA	3.25	0	3.2
ile	ATA	8.12	1.28	4.1
pro	CCC	**29.22**	**20.43**	4.3

[1] Codon frequencies are expressed as codons used per 1,000 codons encountered. The codon frequency of GSTRad9 or MBPRad9, respectively, that differs most from *E.coli* is shown in bold to help identify a codon bias that may cause problems for high-level expression in *E.coli*.

According to this comparison it became clear that the codon frequence of the pro codon CCC was much higher in either GSTRad9 or MBPRad9 compared to *E.coli*. It was therefore reasonable to select an *E.coli BL21-Codon Plus-RP* strain, which includes expression plasmids that contains the tRNA genes proL and argU. The corresponing tRNAs recognize the pro codon CCC and the arg codons AGA/AGG, respectively. So the pro codon seems to be the most critical one in bacterial expression of Rad9 fusion protein (Table 6). To be able to compare the effect of the bacterial expression of recombinant Rad9 protein in this special *E.coli BL21-Codon Plus-RP* strain, a 'conventional' *E.coli* strain, *E.coli –TB1*, was additionally tested in expressing recombinant MBPRad9 protein.

3.2.3. Small scale cultures and solubility check of GSTRad9 and MBPRad9

As described for Rad1 (see above), optimal growth and expression conditions for GSTRad9 and MBPRad9 were established by small-scale culture and solubility check. The expression conditions tested for Rad9 are shown in Table 7.

A_{600} was measured every hour during the induction period (data not shown) and fractions were collected as described for GSTRad1 (see above). For MBP-fusion proteins, LB medium was replaced by rich medium, following manufacturer's instructions.

Table 7. Expression conditions tested for Rad9

GSTRad9 in *E.coli-Codon Plus-RP*		**Expression conditions**	
A_{600}=1	1mM IPTG	LB, 27°C	1h, 2h, 3h, 4h, 5h, 6h
	0.5mM IPTG		
	0.1mM IPTG		
A_{600}=0.5	1mM IPTG		
	0.5mM IPTG		
	0.1mM IPTG		
MBPRad9 in *E.coli-Codon Plus-RP*		**Expression conditions**	
A_{600}=1	0.5mM IPTG	Rich media, 27°C	2h, 3h, 5h
	0.1mM IPTG		
A_{600}=0.5	0.5mM IPTG		
	0.1mM IPTG		
MBPRad9 in *E.coli-TB1*		**Expression conditions**	
A_{600}=1	0.5mM IPTG	Rich media, 27°C	2h, 3h, 5h
	0.1mM IPTG		
A_{600}=0.5	0.5mM IPTG		
	0.1mM IPTG		

3.2.3.1. GSTRad9 expressed in *E.coli BL21-Codon Plus-RP*

Collected fractions were treated as described for GSTRad1. The insoluble fractions were visualised by Coomassie staining (Figure 5A), whereas the soluble fractions were analysed by SDS-PAGE followed by subsequent Coomassie staining (data not shown) or immunoblot analysis (Figure 7A), respectively. Additionally, the soluble fractions were

tested on their binding to Glutathione Sepharose 4B by a protein-minipreparation as described for GSTRad1 and the soluble bound GSTRad9 protein was analysed by SDS-PAGE followed by Coomassie staining (Figure 6A) or immunoblot analysis (data not shown), respectively.

According to the small scale cultures and solubility check of GSTRad9 expressed in *E.coli BL21-Codon Plus-RP,* it can be summerized that GSTRad9 is indeed expressed at a reasonable level, but it is mostly present as an insoluble form and appears in increasing amount in the pellet during the induction period (Figure 5A, not all conditions shown). The soluble fractions analysed showed distinct degradation detected by immunoblot analysis (Figure 7A). Additionally, bacterial expression of GSTRad9 in *E.coli BL21-Codon Plus-RP* was generally leaky. After protein-minipreparation no bound and soluble protein could be detected either by Coomassie stained gel (Figure 6A, not all conditions shown) nor by immunoblot analysis (data not shown).

3.2.3.2. MBPRad9 expressed in *E.coli BL21-Codon Plus-RP*

Small scale cultures were used as described for GST-fusion proteins and different expression conditions were tested as written in Table 7. The insoluble fractions (pellets) were analysed by SDS-PAGE and Coomassie staining (Figure 5B), whereas the soluble fractions (supernatants) were directly used for protein-minipreparation. Thereby, washed and equilibrated Amylose-resin was incubated with the soluble fractions of each expression condition as described above (see Materials and Methods). The bound and soluble protein was then analysed by SDS-PAGE and Coomassie staining (Figure 6B) or immunoblot analysis (Figure 7B), respectively. Comparing these results with GSTRad9 expression in *E.coli BL21-Codon Plus-RP*, it is obvious that the expression level of MBPRad9 is distinctly higher, although the same *E.coli* strain was used. A bigger amount of insoluble protein could be found in the pellet (Figure 5B) and, opposite to GSTRad9, a reasonable amount of soluble protein bound to Amylose-resin in each expression condition tested (Figures 6B, 7B). Thereby the highest amount of soluble bound protein was expressed at A_{600}=0.5, 0.1mM IPTG, 5-7 hrs induction (Figures 6B, 7B), but there was a lot of degraded protein present (Figure 7B). A_{600}=1, 0.5mM IPTG, 3-4 hrs

induction was chosen as the best expression condition, showing little less expressed MBPRad9 protein but also less degradation.

3.2.3.3. MBPRad9 expressed in *E.coli-TB1*

Small scale cultures, solubility check and protein-minipreparation of MBPRad9 in *E.coli-TB1* was done using the same methods as described for MBPRad9 in *E.coli BL21-Codon Plus-RP*. The insoluble fractions (pellets) were analysed by SDS-PAGE followed by Coomassie staining (Figure 5C), whereas the soluble fractions (supernatants) were used for protein-minipreparation as described above and soluble bound protein was analysed as well by SDS-PAGE and Coomassie staining (Figure 6C) or immunoblot analysis (Figure 7C), respectively. It was interesting that both *E.coli-TB1* as well as *E.coli BL21-Codon Plus-RP* expressed MBPRad9 at the same high level (Figures 5B, 5C). On the other hand, the soluble and bound MBPRad9 protein fraction was clearly lower in *E.coli-TB1* (Figures 6C, 7C). Nevertheless, bound and soluble expressed MBPRad9 protein could be found in every expression condition tested. The best condition was A_{600}=0.5, 0.5mM IPTG, 5 hrs induction, although a lot of degraded protein was present.

3.2.4. Optimised expression conditions for Rad9

As a conclusion of all these data from small-scale culture, solubility check and protein-minipreparation, it was decided to express Rad9 as a MBP-fusion protein, because the expression level was clearly improved compared to GSTRad9 protein expression, and soluble and bound recombinant MBPRad9 protein could be found at reasonable levels.
Comparing the two bacterial strains *E.coli BL21-Codon Plus-RP* and *E.coli-TB1*, the first one was clearly superior in expressing soluble recombinant MBPRad9 protein. Based on the observation of increasing degradation at A_{600}=0.5 during induction period, MBPRad9 was expressed in *E.coli BL21-Codon Plus-RP* using following expression conditions: Induction at A_{600}=1 with 0.5mM IPTG and an induction period of 3 hrs at 27°C in rich media.

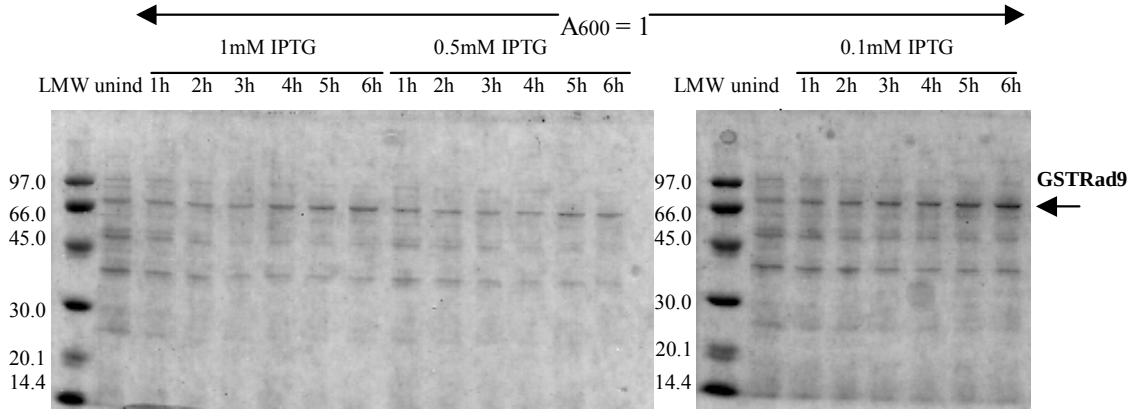

Figure 5A. GSTRad9 expression in *E.coli BL21-Codon Plus-RP*, pellets, Coomassie stained gel. A Coomassie blue-stained 12% SDS-polyacrylamide gel of the insoluble fractions (pellets) of GSTRad9 expressed in *E.coli BL21-Codon Plus-RP* strain is shown. 10µl of each collected 1ml fraction was loaded. *Left side:* Lanes: 1, low molecular weight markers; 2, uninduced cells, 3-8, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 9-14, culture induced at A600=1 with 0.5mM IPTG, 1-6 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells, 3-8, culture induced at A600=1 with 0.1mM IPTG, 1-6 hrs of induction. The position of the GSTRad9 band is indicated on the right.

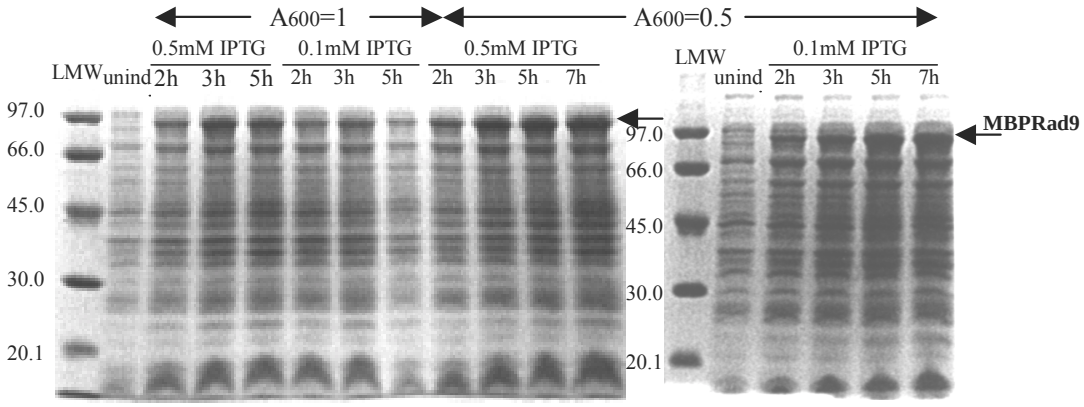

Figure 5B. MBPRad9 expression in *E.coli BL21-Codon Plus-RP*, pellets, Coomassie stained gel. A Coomassie blue-stained 10% SDS-polyacrylamide gel of the insoluble fractions (pellets) of MBPRad9 expressed in *E.coli BL21-Codon Plus-RP* strain is shown. 10µl of each collected 1ml fraction was loaded. *Left side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 2,3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction; 9-12, culture induced at A600=0.5 with 0.5mM IPTG, 2, 3, 5 and 7 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-6, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right.

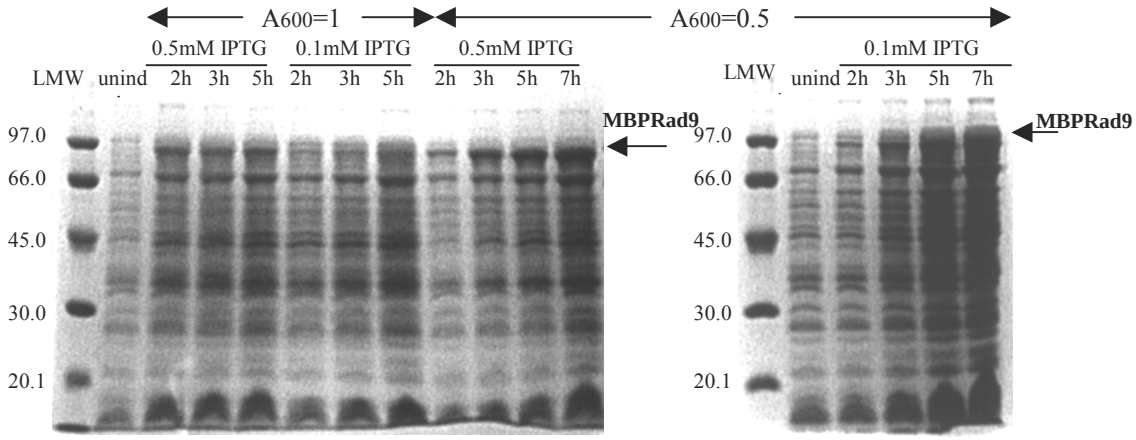

Figure 5C. MBPRad9 expression in *E.coli-TB1*, pellets, Coomassie stained gel. A Coomassie blue-stained 10% SDS-polyacrylamide gel of the insoluble fractions (pellets) of MBPRad9 expressed in *E.coli – TB1* strain is shown. 10µl of each collected 1ml fraction was loaded. *Left side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 2, 3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction; 9-12, culture induced at A600=0.5 with 0.5mM IPTG, 2, 3, 5 and 7 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells, 3-6, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right.

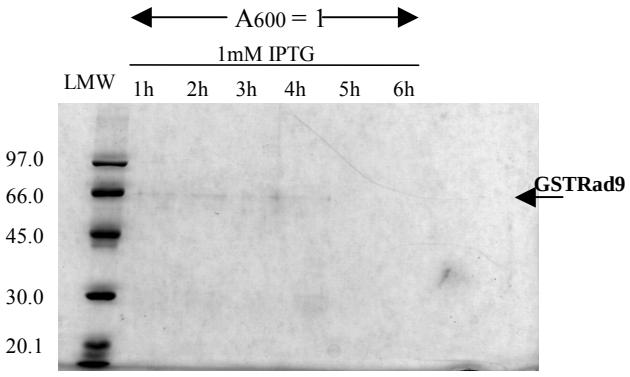

Figure 6A. GSTRad9 expression in *E.coli BL21-Codon Plus-RP*, protein-minipreparation, Coomassie stained gel. The soluble fractions of GSTRad9 expressed in *E.coli BL21-Codon Plus-RP* strain were incubated with washed and equilibrated Glutathione Sepharose 4B 50% slurry . Unbound protein was removed, Glutathione Sepharose 4B beads were washed and protein was eluted as described in Material and Methods. 25µl of the samples containing eluted protein were resolved by 12% SDS-PAGE and stained by Coomassie. Lanes: 1, low molecular weight markers; 2-7, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction. The position of the GSTRad9 band is indicated by an arrow on the right.

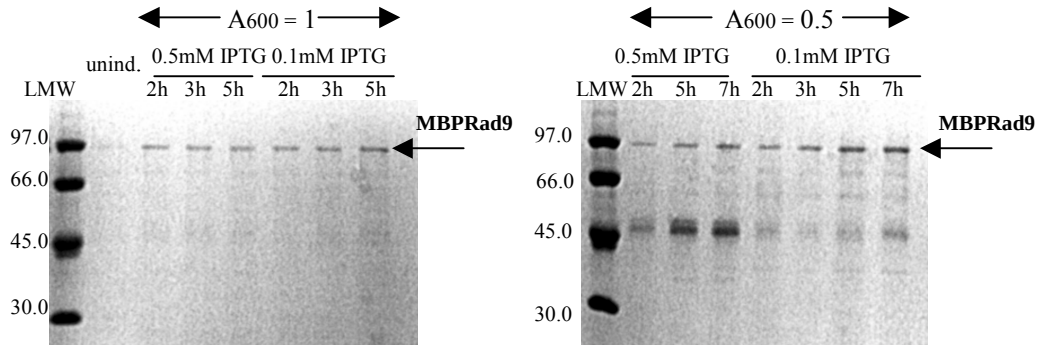

Figure 6B. MBPRad9 expression in *E.coli BL21-Codon Plus-RP*, protein-minipreparation, Coomassie stained gel. The soluble fractions of MBPRad9 expressed in *E.coli BL21-Codon Plus-RP* were incubated with washed and equilibrated amylose–resin 50% slurry. Unbound protein was removed, amylose-resin washed and protein eluted as described above. 25µl of the samples containing eluted protein were resolved by SDS-PAGE and stained by Coomassie. *Left side:* Lanes: 1, low molecular weight markers; 2, uninduced cells, 3-5, culture induced at A600=1 with 0.5mM IPTG, 2, 3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction; *Right side:* Lanes: 1, low molecular weight markers; 2-4, culture induced at A600=0.5 with 0.5mM IPTG, 2, 5 and 7 hrs of induction; 5-8, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right.

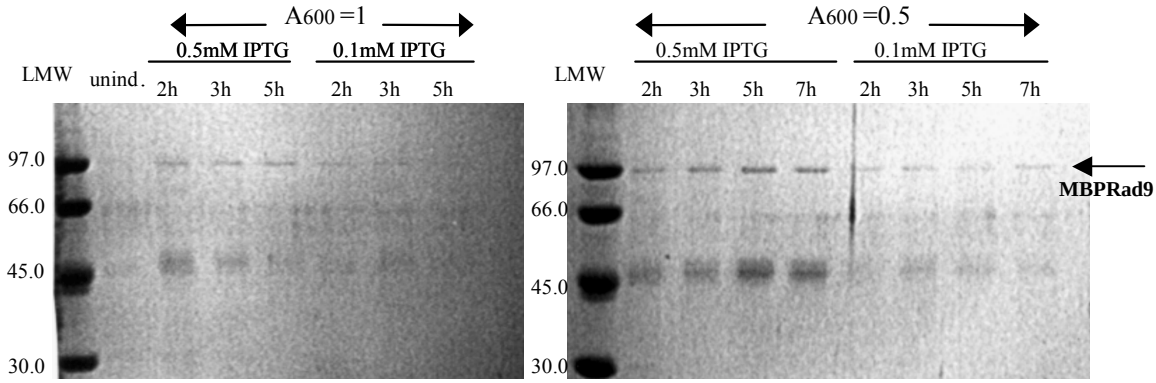

Figure 6C. MBPRad9 expression in *E.coli-TB1*, protein-minipreparation, Coomassie stained gel. The soluble fractions of each collected 1ml aliquot from the MBPRad9 expression in *E.coli-TB1* were treated as described for Figure 6B. 25µl of the samples containing protein eluted from the beads were resolved by 10% SDS-PAGE and stained by Coomassie. *Left side*: Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 2, 3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction; *Right side:* Lanes: 1, low molecular weight markers; 2-5, culture induced at A600=0.5 with 0.5mM IPTG, 2, 3, 5 and 7 hrs of induction; 6-9, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right.

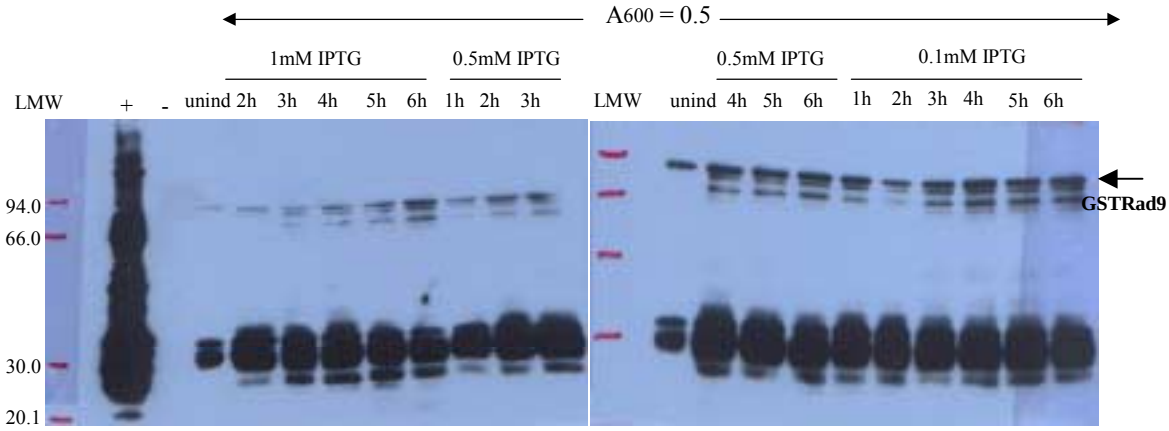

Figure 7A. GSTRad9 expression in *E.coli BL21-Codon Plus-RP*, supernatant, immunoblot analysis. 20µl of the soluble part of GSTRad9 expressed in *E.coli BL21-Codon Plus-RP* were analysed by 12% SDS-PAGE followed by immunoblot analysis using a α-GST polyclonal rabbit antibody. *Left side:* Lanes: 1, low molecular weight markers; 2, positive control GSTRad17; 3, negative control Aldolase; 4, uninduced cells; 5-9, culture induced at A600=0.5 with 1mM IPTG, 2-6 hrs of induction; 10-12, culture induced at A600=0.5 with 0.5mM IPTG, 1-3 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=0.5 with 0.5mM IPTG, 4-6 hrs of induction; 6-11, culture induced at A600=0.5 with 0.1mM IPTG, 1-6 hrs of induction. The GSTRad9 band is indicated on the right.

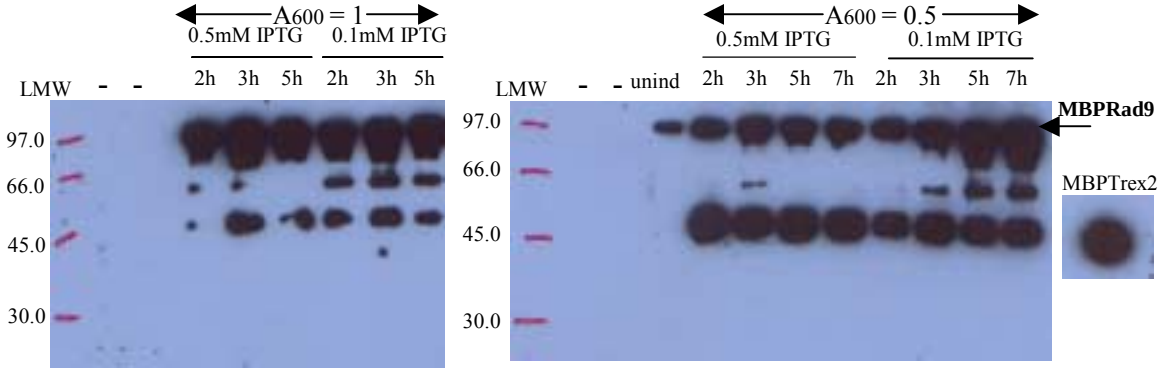

Figure 7B. MBPRad9 expression in *E.coli BL21-Codon Plus-RP*, protein-minipreparation, immunoblot analysis. The fractions containing soluble expressed and bound MBPRad9 protein expressed in *E.coli BL21-Codon Plus-RP* were treated as described for Figure 6B. 25µl of the samples containing eluted protein were resolved by 10% SDS-PAGE and analysed by immunoblot analysis using a α-MBP polyclonal rabbit antibody. *Left side:* Lanes: 1, low molecular weight markers; 2, negative control GSTRad17; 3, negative control Aldolase; 4-6, culture induced at A600=1 with 0.5mM IPTG, 2, 3 and 5 hrs of induction; 7-9, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, negative control; 3, negative control; 4, uninduced cells; 5-8, culture induced at A600=0.5 with 0.5mM IPTG, 2, 3, 5 and 7 hrs of induction; 9-12, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right, followed by the positive control MBPTrex2.

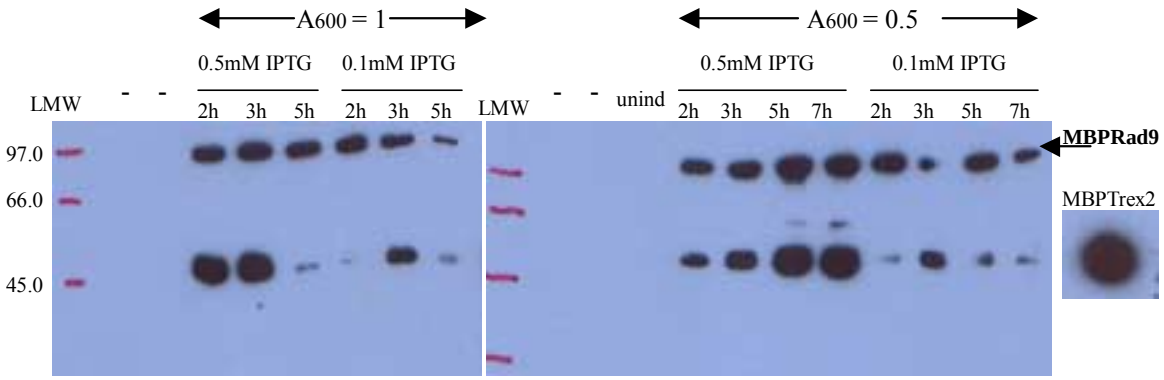

Figure 7C. MBPRad9 expression in *E.Coli-TB1*, protein-minipreparation, immunoblot analysis. The fractions containing soluble and bound MBPRad9 protein expressed in *E.coli-TB1* were treated as described for Figure 6B. 25µl of the samples containing eluted protein were resolved by 10% SDS-PAGE and analysed by immunoblot analysis using a α-MBP polyclonal rabbit antibody. *Left side:* Lanes: 1, low molecular weight markers; 2, negative control GSTRad17; 3, negative control Aldolase; 4-6, culture induced at A600=1 with 0.5mM IPTG, 2, 3 and 5 hrs of induction; 7-9, culture induced at A600=1 with 0.1mM IPTG, 2, 3 and 5 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2, negative control; 3, negative control; 4, uninduced cells; 5-8, culture induced at A600=0.5 with 0.5mM IPTG, 2, 3, 5 and 7 hrs of induction; 9-12, culture induced at A600=0.5 with 0.1mM IPTG, 2, 3, 5 and 7 hrs of induction. The position of the MBPRad9 band is indicated by an arrow on the right, followed by a positive control MBPTrex2.

3.2.5. Big culture and purification of MBPRad9

3.2.5.1. Amylose-resin batch purification of MBPRad9

pMalc-2E-Rad9 plasmid was transformed into *E.coli BL21-Codon Plus-RP* cells. A single colony was inoculated in LB medium and incubated overnight. Overnight culture was then added to flasks containing rich media and incubated to A_{600}=1. IPTG was added to a final concentration of 0.5mM and the culture induced for 3 hrs at 27°C and 250 rpm. Aliquots were collected before (uninduced cells) and after (induced cells) induction. The cells were harvested by centrifugation and resuspended in lysis buffer. The bacteria were lysed by passage through a French press and the lysate was separated by centrifugation into a soluble (supernatant) and insoluble (pellet) fraction. The soluble fraction (load) was added to washed and equilibrated Amylose-resin slurry and rotated for several hours. After centrifugation, the supernatant was removed (unbound protein) and the Amylose-

resin washed several times (wash). Bound recombinant MBPRad9 protein was then eluted six times with elution buffer by rotating each time for several minutes followed by centrifugation and collecting the supernatant (E1-E6). Load, unbound protein, wash and each collected fraction of eluted protein (E1-E6) were analysed by SDS-PAGE and Coomassie staining (Figure 8A).

After this first purification step, a strong band with the predicted molecular mass of 85.4 kDa, representing a high amount of soluble MBPRad9 recombinant protein could be found in the eluted fractions E1-E6 with decreasing amount of eluted MBPRad9 protein during the elution steps. Some MBPRad9 was also present in the unbound and wash fractions, showing the limited capacity of this batch purification system. Purified MBPRad9 recombinant protein was not that pure and showed a distinct second band at about 45 kDa representing either contamination or degraded MBPRad9 protein. According to these results a second purification step was added. Therefore the fractions E1-E6 containing MBPRad9 were pooled. The pI of MBPRad9 recombinant protein was calculated based on its codon sequence as described for GSTRad1. The pI of MBPRad9 was 5.03 and therefore an anion exchange column was chosen as second purification step.

3.2.5.2. Purification of MBPRad9 by an anion exchanger HiTrap Q

The function of an anion exchanger is described above. An important parameter using this purification system is the pH chosen for the starting buffer. It should be, as described before, at least 1unit above the pI of the recombinant protein to be purified ($pI_{MBPRad9}$= 5.03). A lower pH would lead to an insufficient binding of the protein to the column, whereas a too high chosen pH could cause protein degradation. According to the fact that MBPRad9 has exactly the same calculated pI as GSTRad1, it was decided to choose the same pH for buffer A as used for GSTRad1 purification. So enrichment of recombinant MBPRad9 protein was achieved by pH 8 of buffer A, although the binding of GSTRad1 to the anion exchanger was not sufficient using this pH (see above). But a pH above pH 8 would likely cause protein degradation that would be very hard to separate from non-degraded MBPRad9 as known from GSTRad1 purification. So the pooled fractions were dialysed against buffer A (load), and loaded onto the prepacked and equilibrated HiTrapQ

anion exchange column as described above (see Materials and Methods) using a FPLC-system. The flow through was saved and the column was washed several times, saving as well the washing fractions (wash). Bound MBPRad9 recombinant protein was then eluted with a continuous salt gradient elution and fractions were collected. Load, flow through and the collected fractions were analysed by SDS-PAGE and Coomassie staining (Figure 8B). Most of the recombinant MBPRad9 protein eluted from the column between fractions 16-20, represented by a clear band at 85.4 kDa. The second band at about 45 kDa, representing either degraded protein or contamination could be separated by this second purification step and eluted earlier from the anion exchanger column, mainly between fractions 11-14. A second band at about 60 kDa could not be separated properly and eluted between fraction 17-23 from the column, a little bit later than most of MBPRad9 recombinant protein. Contrary to GSTRad1, it can be mentioned that the binding of MBPRad9 to the anion exchanger column was sufficient using a starting buffer with pH 8. No protein could be found in the flow through or washing fractions. Fractions 16-20 containing the main part of MBPRad9 recombinant protein were pooled to a main pool, Pool 1. Fractions 21-30 and 13-15 were pooled to two side pools, Pool 2 and Pool 3, respectively. The protein content of each pool was quantified by Bradford assay (see Discussion, Table 10) and analysed by SDS-PAGE followed by subsequent Coomassie staining (Figure 8C). The pooled fractions were then dialysed against a storage buffer (see Materials and Methods), aliquoted and frozen at –80°C until further use (see Activity test, Figure 12).

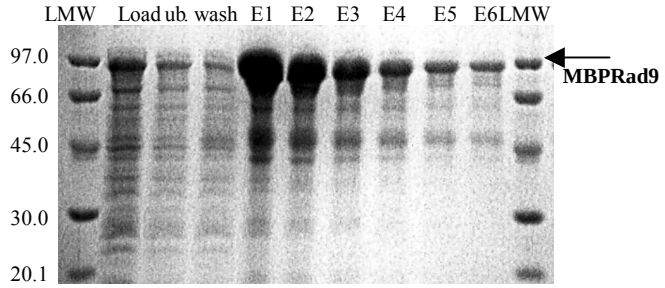

Figure 8A. MBPRad9 purification, 1.6 l culture, Amylose-resin batch purification, Coomassie stained gel. The bacterial pellet of a 1.6 l culture of *E.coli BL21-Codon Plus-RP* expressing MBPRad9 was resuspended, lysed and separated into a soluble and insoluble fraction by centrifugation as described in Materials and Methods. Amylose-resin was washed, equilibrated and incubated with the soluble protein fraction. The unbound protein was removed, the resin washed, bound protein eluted six times and the supernatant containing purified MBPRad9 recombinant protein was collected (E1-E6). Saved aliquots of the load, the unbound protein, the wash and 10µl of each collected fraction E1-E6 were resolved by 10% SDS-PAGE and analysed by Coomassie staining. Lanes: 1, low molecular weight markers; 2, 1µl load: 3, 1µl unbound protein; 4, 17.5µl wash; 5-10, 10µl of each eluted fraction E1-E6; 11, low molecular weight markers. The position of the MBPRad9 band is indicated by an arrow on the right.

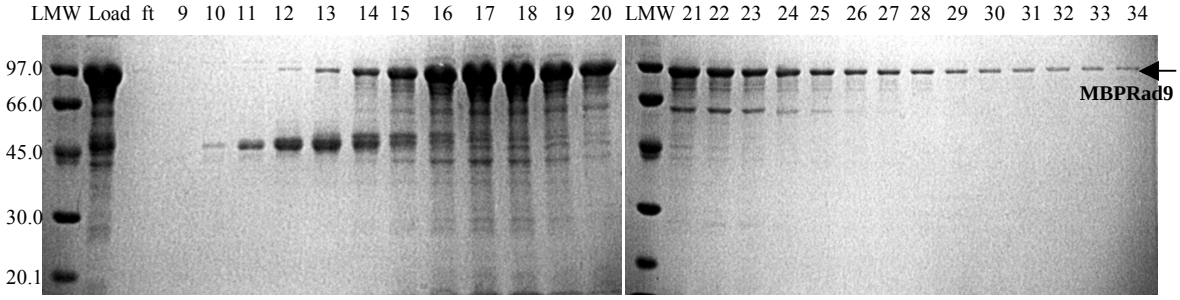

Figure 8B. MBPRad9 purification, anion exchange column HiTrap Q, Coomassie stained gel. The six eluted fractions E1-E6 from 8A. were pooled, dialysed and loaded onto a prepacked and equilibrated HiTrap Q anion exchange column as described in Materials and Methods. The column was washed, the protein eluted and 0.25ml fractions were collected. Aliquots of the load, flow through and 10µl of each collected 0.25ml fraction were analysed by 10% SDS-PAGE followed by Coomassie staining. *Left side:* Lanes: 1, low molecular weight markers; 2, 10µl load; 3, 10µl flow through; 4-15, 10µl of each fraction 9-20 containing MBPRad9 protein eluted from the anion exchange column. *Right side:* Lanes: 1, low molecular weight markers; 2-15, 10µl of each fraction 21-34 containing MBPRad9 protein eluted from the anion exchange column. The position of the MBPRad9 band is indicated by an arrow on the right.

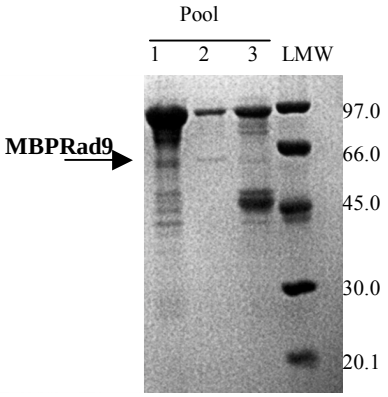

Figure 8C. MBPRad9 purification, Pool 1, Pool 2 and Pool 3 after two-step purification, Coomassie stained gel. After the anion exchange column HiTrap Q, fractions 16-21, containing the main part of MBPRad9 were pooled to a main pool, Pool 1 and analysed by 10% SDS-PAGE followed by Coomassie staining. Two side pools, Pool 2 and Pool 3, containing the fractions 22-30 and 13-15, respectively, were additionally analysed. Lanes: 1, 10µl of Pool 1; 2, 10µl of Pool 2; 3, 10µl of Pool 3; 4, low molecular weight markers. The position of the MBPRad9 band is indicated by an arrow on the left.

3.3. Hus1

3.3.1. Fusion protein and expression conditions

Among the numerous papers published on the function, structure and interactions of Hus1, only two of them describe bacterial expression of this human checkpoint protein (Table 3). Cai *et al.* used a pGEX-2TK expression vector leading to a GST-tagged fusion protein, GSTHus1 (Cai *et al.*, 2000). For that purpose, the plasmid was transformed into bacteria and induced with IPTG. The precise bacterial strain and induction conditions used are not further described. The protein was purified by its binding to Glutathione Sepharose 4B beads and used for an *in vitro* GST pull-down assay to test its interaction with HDAC1, a histone deacetylase. In a second published paper that describes bacterially expressed recombinant Hus1 protein a pET24a+ expression vector was used to get $(His)_6$-Hus1 which was then used to immunize rabbits (Volkmer and Karnitz, 1999). Bacterial strain, expression conditions, induction period and the solubility of purified $(His)_6$-Hus1 were not further described. According to these data it was decided to test the expression of Hus1 as a GST-tagged fusion protein using a pGEX-expression vector as described for Rad1 and Rad9. Therefore Hus1 cDNA was amplified from pREP4-Hus1

by PCR as described above (see Materials and Methods). The PCR product was then digested and inserted into pGEX-4T-3 to obtain the expression vector pGEX-4T-3-Hus1.

3.3.2. Bacterial strain

First of all the codon frequencies of GSTHus1, a 58 kDa protein, were checked to judge the problem of codon bias expressing this recombinant protein in *E.coli* (Table 8).

Table 8. Codon frequencies of critical codons in GSTHus1 and *E.coli* [1]

Amino-acid	Codon	GSTHus1	*E.coli*
arg	AGG	**5.9**	1.4
	AGA	5.9	2.1
	CGA	7.9	3.1
leu	CTA	9.9	3.2
ile	ATA	**15.7**	4.1
pro	CCC	9.9	4.3

[1] Codon frequencies are expressed as codons used per 1,000 codons encountered. The two codon frequencies of GSTHus1 that differ most from *E.coli* are shown in bold to help identify a codon bias that may cause problems for high-level expression in *E.coli*.

Focussing on the most critical codons like arg, leu, ile and pro, all of them are a little more frequently present in GSTHus1 than in originally expressed *E.coli* protein. The most distinct difference can be found in the arg codon AGG and the ile codon ATA so that the most reasonable *E.coli* strain to use for the expression of GSTHus1 would be *E.coli BL21-Codon Plus-RIL*, which contains extra copies of the argU, ileY and leuW tRNA genes. Nevertheless, these differences were never as significant as calculated for Rad9 or Rad1 recombinant protein (Tables 4, 6). Considering this fact it can be supposed that codon bias would probably not be the main obstacle for the expression of recombinant GSTHus1 protein in *E.coli* and it could therefore be reasonable to test additional strains, for example *E.coli-TB1* or *E.coli-TG1*. Paying regard to their different genetic background it would thereby be possible to test its influence on the expression of soluble recombinant GSTHus1 protein (see Introduction and Problem). Therefore it was

decided to express GSTHus1 in three different *E.coli* strains: *E.coli BL21-Codon Plus-RIL, E.coli-TB1* and *E.coli-TG1*.

3.3.3. Small scale cultures and solubility check of GSTHus1

The three bacterial strains *E.coli BL21-Codon Plus-RIL, E.coli-TB1* and *E.coli-TG1* were tested under different conditions to find out how different IPTG-concentrations, various expression periods and induction at different cell densities would influence the expression level and solubility of recombinant GSTHus1 protein (Table 9).

Table 9. Expression conditions tested for Hus1

GSTHus1 in *E.coli BL21-Codon Plus-RIL*		**Expression conditions**	
A_{600}=1	1mM IPTG	LB, 27°C	1h, 2h, 3h, 4h, 5h, 6h
	0.5mM IPTG		
	0.1mM IPTG		
A_{600}=0.5	1mM IPTG		
	0.5mM IPTG		
	0.1mM IPTG		
GSTHus1 in *E.coli-TB1*		**Expression conditions**	
A_{600}=1	0.5mM IPTG	LB, 27°C	1h, 3h, 5h
	0.1mM IPTG		
A_{600}=0.5	0.5mM IPTG		
	0.1mM IPTG		
GSTHus1 in *E.coli-TG1*		**Expression conditions**	
A600=1	0.5mM IPTG	LB, 27°C	1h, 3h, 5h
	0.1mM IPTG		
A_{600}=0.5	0.5mM IPTG		
	0.1mM IPTG		

Small scale cultures and solubility check were done as described for GSTRad1 (see 3.1.3.), collecting fractions every 1-2 hrs and thereby measuring A_{600} to test the toxicity of expressed GSTHus1 protein on the bacterial cells (data not shown). The insoluble

fractions were visualised by Coomassie stained gels (Figure 9), whereas the soluble fractions were analysed by SDS-PAGE followed by subsequent Coomassie staining (data not shown) or immunoblot analysis (Figure 10), respectively.

3.3.3.1. GSTHus1 expressed in *E.coli BL21-Codon Plus-RIL*

Considering the insoluble fraction (pellet) of the different expression conditions tested, a clear detectable band at the predicted molecular mass of 58 kDa could be identified on Coomassie stained gels representing GSTHus1 (Figure 9A). It got significantly stronger during the expression period, corresponding to the measured increase of A_{600} during expression (data not shown). On the other hand, considering the soluble fraction (supernatant) of GSTHus1 expressed in *E.coli BL21-Codon Plus-RIL*, no band could be identified at the predicted molecular mass, neither on Coomassie stained gel nor by immunoblot analysis (data not shown). This leads to the conclusion that GSTHus1 is indeed expressed in *E.coli BL21-Codon Plus-RIL* at a reasonable level showing no manifest toxicity on the bacterial cells, but that the expressed protein is exclusively present in an insoluble form in the pellet forming so called inclusion bodies (see Introduction and Problem). They represent a denatured form of expressed protein which is therefore not suitable for any biochemical or structural studies. Therefore, the expression of recombinant GSTHus1 in *E.coli BL21-Codon Plus-RIL* was not further analysed.

3.3.3.2. GSTHus1 expressed in *E.coli-TB1*

Small scale cultures and solublity check were performed as described above, testing the different expression conditions written in Table 9. In contrast to the expression in *E.coli BL21-Codon Plus-RIL,* only the two lower IPTG concentrations (0.5mM IPTG, 0.1mM IPTG) were tested for *E.coli-TB1* considering the fact that a low IPTG concentration causes a lower expression level of recombinant protein and can therefore be helpful to get a higher amount of soluble expressed protein (see Introduction and Problem). Looking at the insoluble fractions of GSTHus1 expressed in *E.coli-TB1*, a distinct band at the predicted molecular mass of 58 kDa could be found representing bacterially expressed GSTHus1 recombinant protein (Figure 9B). The expression level in *E.coli-TB1* was even

better than detected for *E.coli BL21-Codon Plus-RIL* although the measured A_{600} wasn't significantly higher, suggesting that the expression capacity per cell seemed to be better in *E.coli-TB1* than in *E.coli BL21-Codon Plus-RIL.* At this stage the soluble fractions of GSTHus1 expressed in *E.coli-TB1* were compared. A clear band at about 58 kDa was detectable by immunoblot analysis (Figure 10A) representing soluble expressed GSTHus1 recombinant protein, whereas the second band at about 27 kDa likely represents GST-protein alone. This supposition is supported by the fact that this 27 kDa band gets distinctly stronger during the induction period. Additionally, it was evident that the amount of soluble expressed GSTHus1 protein was increased when the cells were induced at a lower cell density, supporting the thesis that a lower expression level could in fact lead to a higher amount of soluble protein (see Introduction and Problem). The expression condition chosen for an optimal bacterial expression of GSTHus1 in *E.coli-TB1* was A_{600}=0.5, 0.5mM IPTG, 5 hrs of induction at 27°C.

3.3.3.3. GSTHus1 expressed in *E.coli-TG1*

The small scale cultures and solubility check were done as described above and the same expression conditions were tested for *E.coli-TG1* as well as for *E.coli-TB1* (Table 9). Comparing now the insoluble fractions (pellets) of expressed GSTHus1 in *E.coli-TG1* (Figure 9C) it can be summarized that the recombinant protein is expressed at a reasonable level comparable to *E.coli BL21-Codon Plus-RIL,* but at a lower level than in *E.coli-TB1.* Considering the soluble fractions (Figure 10B) it became clear that only very weak bands of expressed recombinant GSTHus1 at some expression conditions tested could be detected by immunoblot analysis.

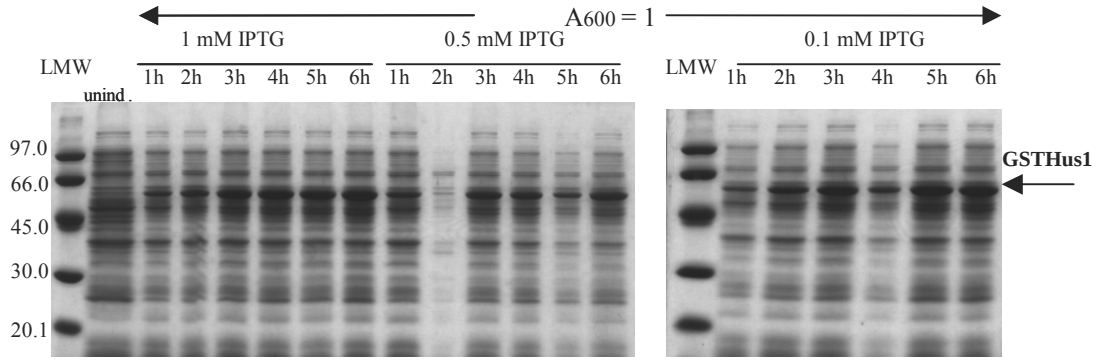

Figure 9A. GSTHus1 expression in *E.coli BL21-Codon Plus-RIL*, pellets, Coomassie stained gels. A Coomassie blue-stained 12% SDS-polyacrylamide gel of the insoluble fractions (pellets) of GSTHus1 expressed in *E.coli BL21-Codon Plus-RIL* strain is shown. 10µl of each collected 1ml fraction was loaded. *Left side:* Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-8, culture induced at A600=1 with 1mM IPTG, 1-6 hrs of induction; 9-14, culture induced at A600=1 with 0.5mM IPTG, 1-6 hrs of induction. *Right side:* Lanes: 1, low molecular weight markers; 2-7, culture induced at A600=1 with 0.1mM IPTG, 1-6 hrs of induction. The position of the GSTHus1 band is indicated by an arrow on the right.

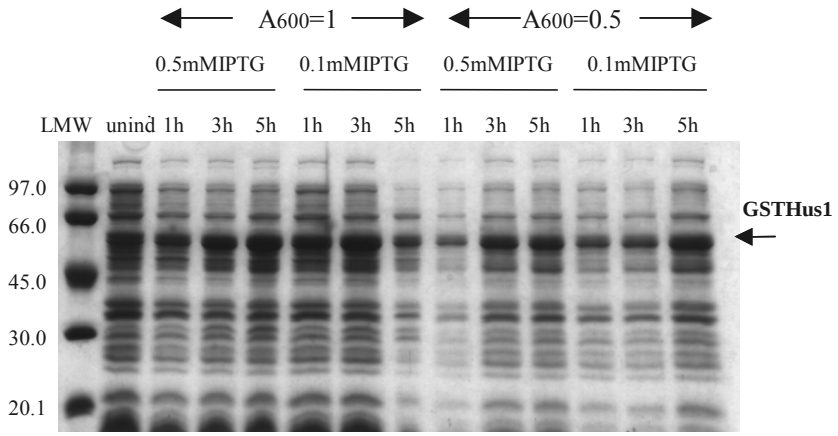

Figure 9B. GSTHus1 expression in *E.Coli-TB1*, pellets, Coomassie stained gel. A Coomassie blue-stained 12% SDS-polyacrylamide gel of the insoluble fractions (pellets) of GSTHus1 expressed in *E.coli-TB1*strain is shown. 10µl of each collected 1ml fraction was loaded. Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 1, 3 and 5 hrs of induction; 9-11, culture induced at A600=0.5 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 12-14, culture induced at A600=0.5 with 0.1mM IPTG, 1, 3 and 5 hrs of induction. The position of the GSTHus1 band is indicated by an arrow on the right.

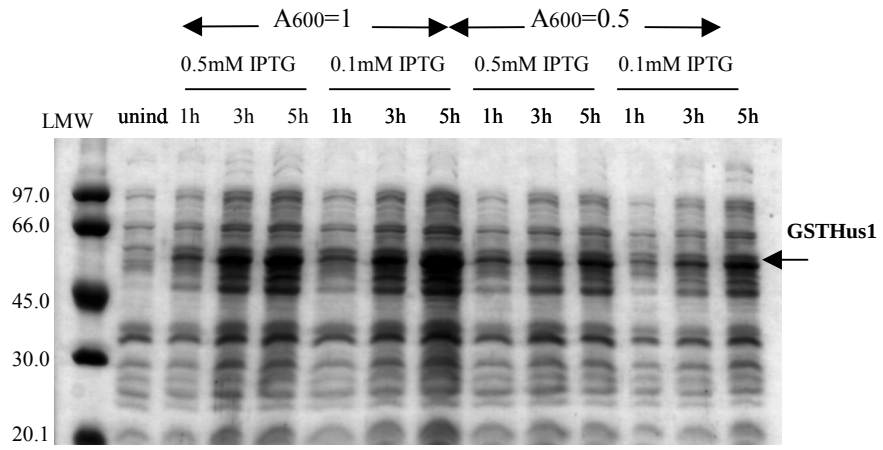

Figure 9C. GSTHus1 expression in *E.coli-TG1*, pellets, Coomassie stained gel. A Coomassie blue-stained 12% SDS-polyacrylamide gel of the insoluble fractions (pellets) of GSTHus1 expressed in *E.coli–TG1* strain is shown. 10µl of each collected 1ml fraction was loaded. Lanes: 1, low molecular weight markers; 2, uninduced cells; 3-5, culture induced at A600=1 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 6-8, culture induced at A600=1 with 0.1mM IPTG, 1, 3 and 5 hrs of induction; 9-11, culture induced at A600=0.5 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 12-15, culture induced at A600=0.5 with 0.1mM IPTG, 1, 3 and 5 hrs of induction. The position of the GSTHus1 band is indicated by an arrow on the right.

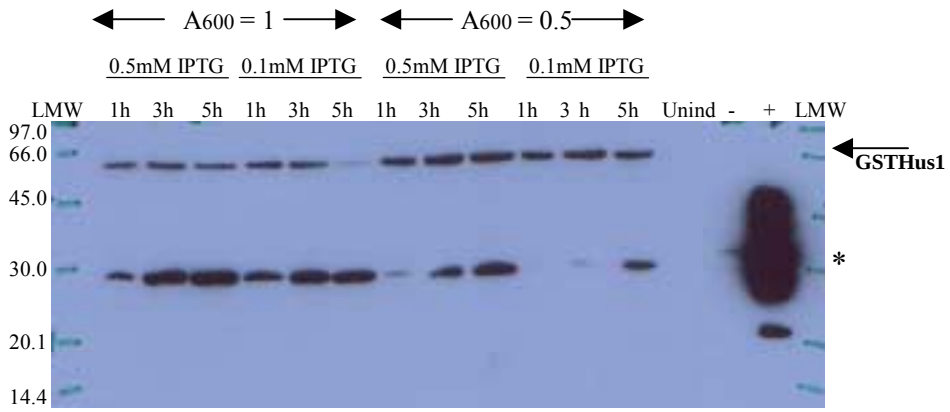

Figure 10A. GSTHus1 expression in *E.coli-TB1*, supernatants, immunoblot analysis. 20µl of the soluble fractions of each aliquot of GSTHus1 expressed in *E.coli-TB1* was analysed by SDS-PAGE followed by immunoblot analysis using a α-GST polyclonal rabbit antibody. Lanes: 1, low molecular weight markers; 2-4culture induced at A600=1 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 5-7, culture induced at A600=1 with 0.1mM IPTG, 1, 3 and 5 hrs of induction; 8-10, culture induced at A600=0.5 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 11-13, culture induced at A600=0.5 with 0.1mM IPTG, 1, 3 and 5 hrs of induction; 14, uninduced cells; 15, negative control Aldolase; 16, positive control GSTRad17; 17, low molecular weight markers. The position of the GSTHus1 band is indicated by an arrow on the right. Degradation is indicated by *.

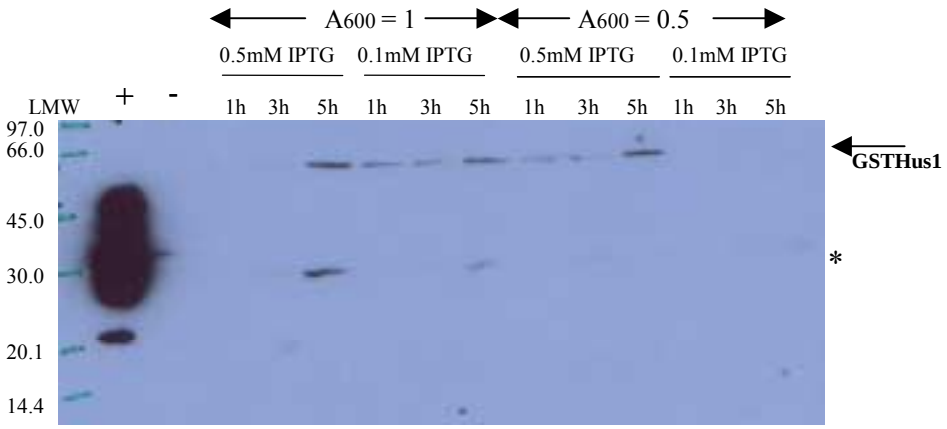

Figure 10B. GSTHus1 expression in *E.coli-TG1*, supernatant, immunoblot analysis. The fractions were treated and analysed as described for 10A.. Lanes: 1, low molecular weight markers; 2, positive control GSTRad17; 3, negative control Aldolase; 4-6, culture induced at A600=1 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 7-9, culture induced at A600=1 with 0.1mM IPTG, 1, 3 and 5 hrs of induction; 10-12, culture induced at A600=0.5 with 0.5mM IPTG, 1, 3 and 5 hrs of induction; 13-15, culture induced at A600=0.5 with 0.1mM IPTG, 1, 3 and 5 hrs of induction. The position of the GSTHus1 band is indicated by an arrow on the right. Degradation is indicated by * .

3.3.4. Optimised expression conditions for Hus1

As a conclusion of all these data, it was evident that *E.coli-TB1* was superior in expressing GSTHus1 recombinant protein in comparison to the two other *E.coli* strains tested. Using this strain, soluble and non-degraded protein could be found in every condition tested, although a lower cell density (A_{600}=0.5) at the point of induction seemed to increase the level of soluble expressed GSTHus1 protein. The distinct second band detected, likely representing GST-protein alone, could probably be separated by a second purification step after Glutathione Sepharose 4B. Nevertheless, to get a reasonable amount of soluble purified GSTHus1 protein, a very large culture must be used for expression. Comparing the three checkpoint proteins Rad1, Rad9 and Hus1, this third human checkpoint protein seemed to be definitely the most difficult one to establish bacterial expression and purification because of its property to appear almost exclusively as inclusion bodies in the pellet.

3.3.5. Big culture and purification of GSTHus1 in *E.coli-TB1*

3.3.5.1. Purification of GSTHus1 by a GSTrap column

pGEX-4T-3-Hus1 plasmid was transformed in *E.coli-TB1* cells following manufacturer's instructions. A single transformed colony was inoculated in LB medium which was then expanded to a 4 l culture. At A_{600}=0.5, IPTG was added to a final concentration of 0.5mM and the culture was incubated at 27°C and 250 rpm for 5hrs. Aliquots were taken from uninduced and induced cells. After 5 hrs of induction the cells were harvested by centrifugation and resuspended in lysis buffer. Bacteria were lysed by passage through a French press and separated into a soluble (supernatant) and insoluble fraction (pellet) by centrifugation. The filtered soluble fraction was then applied to a prepacked and equilibrated GSTrap column using a FPLC-system. An aliquot of the load and the flow through was saved before the column was washed several times (wash). The bound GSTHus1 protein was eluted with elution buffer and fractions were collected. Load, flow through, wash and the collected fractions were analysed by SDS-PAGE and Coomassie staining (Figure 11A). Soluble GSTHus1 recombinant protein eluted mostly between fractions 5 –14, detectable as a band at about 58 kDa. In addition to this 58 kDa form, a second distinct band was present at about 27 kDa. As described for the small scale cultures, this second band seemed to represent GST-protein alone, supported additionally by the fact that it clearly bound to Glutathione Sepharose 4B and eluted at the same fractions as GSTHus1 recombinant protein. Additionally, a third band was distinctly present just below the 58 kDa band, probably representing degraded GSTHus1 protein. This degraded protein was not present in the solubility check of the small scale cultures, probably indicating again that the purification of GST-tagged protein by its binding to Glutathione Sepharose 4B can lead to degradation. This degradation was also observed after the first purification step of GSTRad1, although no degraded protein was found in the analysed supernatant before purification. However, a second purification step of recombinant GSTHus1 protein was necessary.

3.3.5.2. Purification of GSTHus1 by an anion exchanger HiTrap Q

The pI of GSTHus1 based on its codon sequence was calculated as pI=5.03. Therefore an anion exchanger HiTrap Q column was chosen as a second purification step as described for GSTHus1 and MBPRad9. For the starting buffer A pH=8 was selected to achieve a sufficient enrichment of target protein. Fractions 5-14 containing GSTHus1 recombinant protein were pooled and dialysed against buffer A before it was loaded onto the anion exchange column HiTrap Q using a FPLC-system. Bound protein was then eluted with a continuous salt gradient at an increase in ionic strength. Fractions were collected and load, flow through, wash and the collected fractions were analysed by SDS-PAGE and Coomassie staining (Figure 11B). The binding of GSTHus1 recombinant protein to the anion exchange column was sufficient and no target protein could be detected in the flow through and wash fraction. By this second purification step it was possible to remove the 27 kDa band from the fractions containing the main part of GSTHus1. On the other hand, the second contaminating band just below the first 58 kDa band, likely representing degraded GSTHus1 protein, was not separated by this second column. Paying regard to the fact that these two bands eluted at exactly the same point from the column, it seemed to be impossible to separate them by an anion exchange column using different binding and elution conditions.

The fractions 7, 8 and 9, containing most of purified GSTHus1 recombinant protein, were pooled to a main pool, Pool 1, whereas fractions 6,10,11 and 12-22 were pooled to two side pools, Pools 2 and Pool 3, respectively. The protein content of each pool was quantified by Bradford assay (see Discussion, Table 10) and analysed by SDS-PAGE followed by subsequent Coomassie staining (Figure 11C) or immunoblot analysis (data not shown), respectively. The pools were then dialysed against a storage buffer and frozen at –80°C until further use (see Activity test, Figure 12).

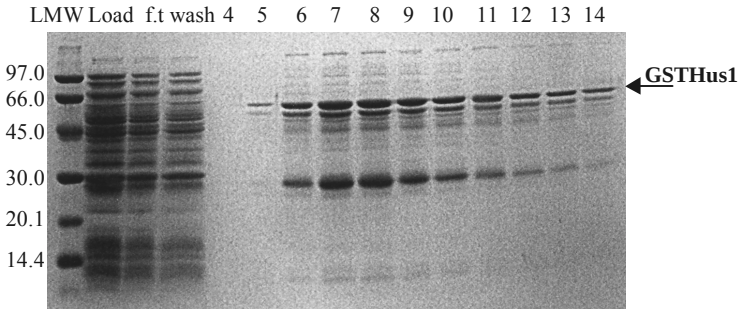

Figure 11A. GSTHus1 purification , 4 l culture, GSTrap column, Coomassie stained gel. The bacterial pellet of a 4 l culture of *E.coli-TB1* expressing GSTHus1 was resuspended, lysed and the soluble fraction filtered as described in Materials and Methods. The filtered supernatant was applied to an equilibrated GSTrap column, followed by several washing steps. Bound GSTHus1 protein was eluted from the column as described above. 0.25ml fractions were collected and 1µl load, 1µl flow through , 1µl wash and 14µl of each collected fraction were resolved by 12% SDS-PAGE and analysed by Coomassie staining. Lanes: 1, low molecular weight markers; 2, load; 3, flow through; 4, wash; 5-15, 14µl of each fraction 4-14 containing GSTHus1 protein eluted from the GSTrap column. The position of the GSTHus1 band is indicated by an arrow on the right.

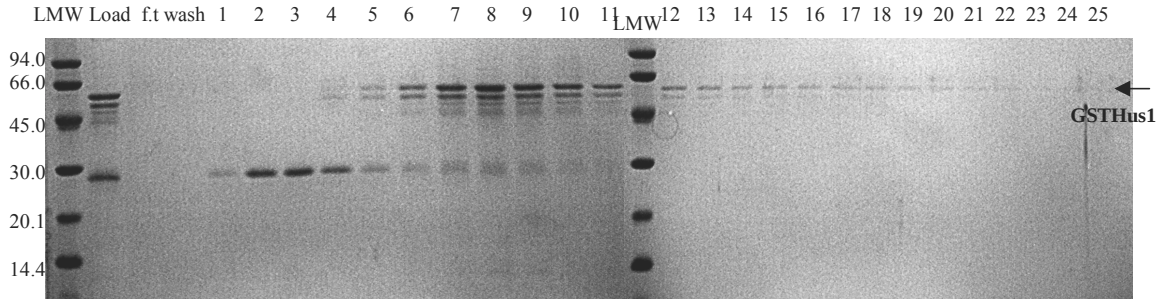

Figure 11B. GSTHus1 purification, anion exchange column HiTrap Q, Coomassie stained gel. Pooled and dialysed fractions containing the main part of GSTHus1 after GSTrap were loaded onto a prepacked and equilibrated HiTrap Q anion exchange column as described in Materials and Methods. After several washing steps, the protein was eluted and 0.25ml fractions were collected. Aliquots of the load, flow through and wash were analysed together with 10µl of each collected 0.25ml fraction by 12% SDS-PAGE followed by Coomassie staining. *Left side:* Lanes: 1, low molecular weight markers; 2, 10µl load; 3, 10µl flow through; 4, 10µl wash; 5-15, 10µl of each fraction 1-11 containing GSTHus1 protein eluted from the anion exchange column. *Right side:* Lanes: 1, low molecular weight markers; 2-15, 10µl of each fraction 12-25 containing GSTHus1 protein eluted from the anion exchange column. The position of the GSTHus1 band is indicated by an arrow on the right.

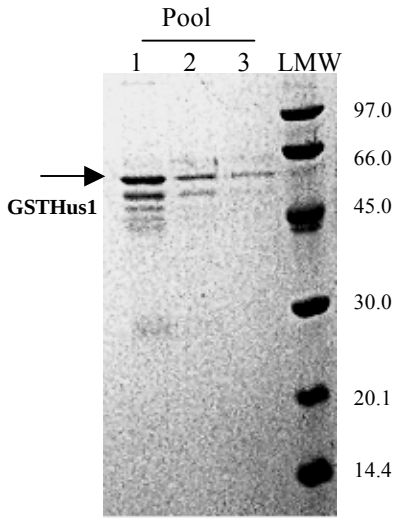

Figure 11C. GSTHus1 purification, Pool 1, Pool 2 and Pool 3 after two-step purification, Coomassie stained gel. After the anion exchange column HiTrap Q, fractions 7-9, containing the main part of GSTHus1 were pooled to a main pool, Pool 1 and analysed by 12% SDS-PAGE followed by Coomassie staining. Two side pools, Pool 2 and Pool 3, containing the fractions 6, 10, 11 and 12-22, respectively, were additionally analysed. Lanes: 1, 10µl of Pool 1; 2, 10µl of Pool 2; 3, 10µl of Pool 3; 4, low molecular weight markers. The position of the GSTHus1 band is indicated by an arrow on the left.

3.4. Activity test of purified GSTRad1, MBPRad9 and GSTHus1

After having optimised the expression conditions for Rad1, Rad9 and Hus1 in small scale cultures and the expression and purification of a reasonable amount of soluble protein in a large scale culture it was important to prove that all three purified recombinant proteins were properly folded. As described earlier (see Introduction and Problem), molecular modelling studies support the thesis that Rad9, Rad1 and Hus1 form a heterotrimeric ring similar to PCNA (Figure 1). In later experiments, Rad9, Rad1 and Hus1 have been shown to interact by yeast-two-hybrid analysis and immunoprecipitation (Caspari *et al.*, 2000; Hang and Lieberman, 2000; St Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Based on these data, functional activity of the three checkpoint proteins could thereby be proved by testing their direct interaction *in vitro*. Dot blot Far Western analysis was therefore performed to determine whether purified recombinant MBPRad9 and GSTHus1 or MBPRad9 and GSTRad1, respectively, showed direct and specific interaction *in vitro*. Essentially, this procedure involves the immobilisation of one of the proteins of interest to a nitrocellulose filter which is then incubated in buffer containing the second protein. The filter is washed to remove any unbound material and the presence of the second protein is detected by conventional Western analysis.

As a first step, three increasing amounts of purified MBPRad9 protein were dotted onto a nitrocellulose filter. Additionally, wtPCNA and MBPTrex2 used as negative controls and GSTRad17 and GSTHus1 as positive controls were immobilised on the same nitrocellulose filter. The nitrocellulose filter was dried on air, then rehydrated in aqua bidest and finally incubated with either purified GSTHus1 or GSTRad1. Conventional Western analysis was then used to detect the presence of GSTHus1 or GSTRad1 using α-GST polyclonal antibody (Figure 12).

When GSTHus1 was used to probe MBPRad9 bound to nitrocellulose, three immunoreactive dots of increasing size could be detected at the position where MBPRad9 had been immobilised. This was due to Hus1 binding to Rad9, as opposed to crossreactivity of α-GST antibody with MBPRad9, because this dot was absent on a control blot with immobilised GSTRad17, MBPTrex2 and MBPRad9 that had been incubated in TBS without Hus1 or Rad1, respectively, but had otherwise been treated in the same way. The interaction between Hus1 and Rad9 appeared to be specific because Hus1 did not bind to either of two control proteins, MBPTrex2 and wtPCNA, which were immobilised in parallel on the same blot. The same experiment was carried out wherein GSTRad1 was used to probe nitrocellulose-bound MBPRad9. Three immunoreactive dots of increasing size could again be detected at the position where MBPRad9 had been immobilised. This interaction between Rad1 and Rad9 also seemed to be specific because Rad1 did not bind to either of the two control proteins MBPTrex2 and wtPCNA, which were immobilised on the same blot. It can be concluded that the purified recombinant Rad9, Hus1 and Rad1 were most likely properly folded since they could interact with each other in a so called “Dot blot Far Western” analysis (Figure 12), suggesting that they might be functionally expressed in the bacterial expression systems used in this study.

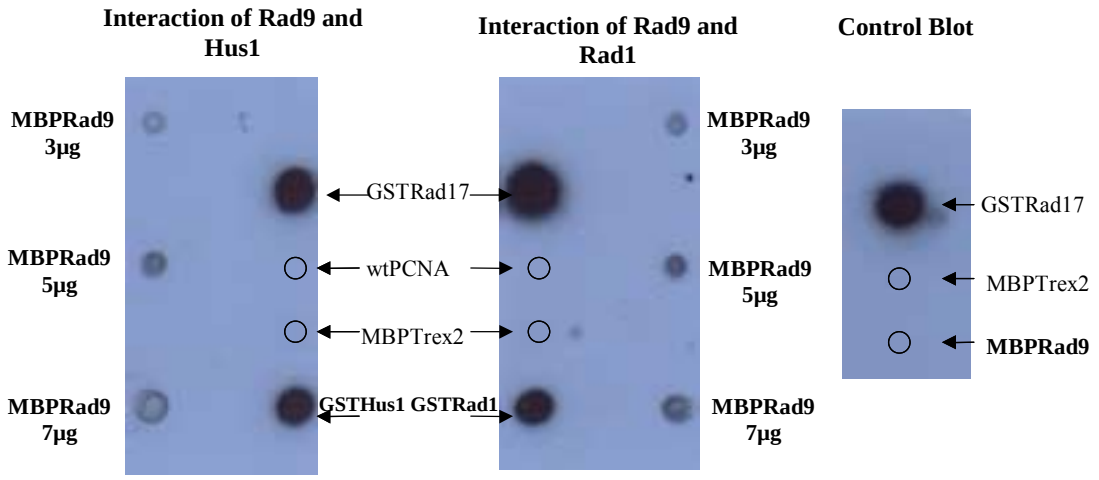

Figure 12. Interaction of purified Rad9, Rad1 and Hus1 *in vitro*. Three increasing amounts of purified MBPRad9 protein were dotted onto a nitrocellulose filter. wtPCNA and MBPTrex2, used as negative controls and GSTRad17 and GSTHus1 as positive controls were immobilised on the same nitrocellulose filter. The filter was dried on air, rehydrated and incubated with either purified GSTHus1 or GSTRad1. Conventional Western blot analysis was used to detect the presence of GSTHus1 or GSTRad1 using α-GST polyclonal antibody. Three immunoreactive dots of increasing size could be detected at the position where MBPRad9 have been immobilised, due to Rad9 binding to Hus1 or Rad1, respectively. *Left blot:* Lane 1: 3µg, 5µg and 7µg of purified MBPRad9 recombinant protein. Lane 2: 2µl GSTRad17, 3µl wtPCNA, 2µl MBPTrex2; 0.5µg GSTHus1. *Middle blot:* Lane 1: 2µl GSTRad17, 3µl wtPCNA, 2µl MBPTrex2; 2µl GSTRad1. Lane 2: 3µg, 5µg and 7µg of purified MBPRad9 recombinant protein. *Control blot:* 2µl GSTRad17, 2µl MBPTrex2, 3µg MBPRad9.

4. Discussion

4.1. Optimised bacterial expression of Rad1

Among the three checkpoint proteins analysed, Rad1 appeared to be the most suitable for its expression in *E.coli*, although only very little data is so far published on bacterial expression of recombinant Rad1 protein. Expressing it as a GST-fusion protein resulted in very good yields of soluble protein (Table 10). According to the distinct differences between the codon frequencies of GSTRad1 and *E.coli* (Table 4), the expression in special *E.coli BL21-Codon Plus-RIL* strains seemed to be reasonable and resulted in high

yields of expression in every condition tested. Thereby, a time-course analysis of the expression level used to check the soluble and insoluble fractions showed indeed that GSTRad1 was mainly expressed as an insoluble form, but that about 25% of the expressed protein was nevertheless present in the soluble fractions. This soluble protein expressed was moreover present in a non-degraded form and at a constant level during the induction period. Thereby, the different expression conditions and the various induction periods tested showed no distinct differences in the expression level of soluble, non-degraded protein. On the other hand, it could be shown by protein-minipreparation that the soluble GSTRad1 recombinant protein was able to bind to Glutathione Sepharose 4B, but thereby showed some degradation which got clearly stronger during the induction period. According to these results, GSTRad1 was expressed in *E.coli BL21-Codon Plus-RIL* in a large scale culture. Therefore, a short induction period (3 hrs), a high concentration of IPTG (1mM) and a high cell density at the time of induction (A_{600} =1) was chosen resulting in a short but high level protein expression with less degradation. The purification of this large-scale culture in two steps using a GSTrap and an anion exchange column led to a reasonable amount of 1.3mg/ml soluble purified GSTRad1 recombinant protein (Table 10), although the conditions chosen for the anion exchanger HiTrap Q did not result in a sufficient binding of GSTRad1 to the column and need to be optimised. Finally, the purified Rad1 recombinant protein was shown to interact directly with Rad9 *in vitro* and therefore appeared to be properly folded.

4.2. Optimised bacterial expression of Rad9

Comparing the data published on the three checkpoint proteins, Rad9 seemed to be the best characterized one and numerous papers exist about its function, structure and interaction with other proteins. Therefore, two published papers could be taken as basis for the experiments that mentioned bacterially expressed MBPRad9 and GSTRad9 recombinant protein in functional assays but without any details about the precise bacterial strain and expression conditions used (Schwartz *et al.*, 2002; Bessho and Sancar, 2000). Considering the distinct differences between the codon frequencies of Rad9 fusion proteins and *E.coli,* this checkpoint protein was very suitable for its expression in *E.coli BL21-Codon Plus-RP* strain. Testing additionally a 'conventional' *E.coli-TB1* strain, the

expression level and the solubility of expressed recombinant protein could be compared. Using a time-course analysis to check the expression level of the soluble and insoluble fraction at various times after induction, it was shown that the bacterial expression of Rad9 as a MBP-fusion protein was clearly superior to its expression as a GST-fusion protein. This confirms the results of earlier systematic comparisons of different fusion partners in increasing the solubility of passenger polypeptides (Kapust and Waugh, 1999). It was thereby found that MBP was far superior to either thioredoxin or Glutathione-S-transferase (GST) as a solubilizing partner. In our studies, it was additionally shown that not only the solubilizing effect was obvious but also the expression level could be clearly increased. At this point, the ability of the two strains *E.coli BL21-Codon Plus-RP* and *E.coli-TB1* in expressing MBPRad9 recombinant protein was compared. The expression level and the amount of soluble protein expressed in *E.coli BL21-Codon Plus-RP* was far superior to *E.coli-TB1,* although exactly the opposite was observed for GSTHus1. This could be due to the fact that the high number of pro codons CCC in the codon sequence of Rad9 plays in fact a critical role. This so called codon bias seemed to be a distinct obstacle for the efficient expression of recombinant Rad9 protein in *E.coli.* Therefore, the expression of soluble recombinant MBPRad9 protein can be clearly increased using *E.coli BL21-Codon Plus-RP* strains. Finally, when comparing the time-course analysis and the different expression conditions tested, it can be summarized that a high cell density at the time of induction (A_{600} =1), a quite high IPTG concentration (0.5mM) and a short induction period (3hrs) was able to minimize protein degradation. Using these optimised expression conditions for a large-scale culture followed by a two step purification, an amount of 0.99mg/ml soluble purified MBPRad9 protein was reached (Table 10). Thereby, the described amylose-resin batch purification was shown to bind a high amount of soluble MBPRad9 protein, although the purity of bound MBPRad9 after this first purification step was not sufficient. On the other hand, a second purification step using an anion exchange column HiTrap Q could clearly separate some contamination and thereby increased the amount of homogeneous and soluble MBPRad9 protein. Although further purification steps could be required, the most likely proper folding of Rad9 after these two steps of purification was shown by its direct and specific interaction with Hus1 and Rad1 *in vitro*.

4.3. Optimised bacterial expression of Hus1

As opposed to Rad9, only little data is published on Hus1 which seemed to be the least characterized of the three checkpoint proteins examined. Nevertheless published data was available on bacterially expressed GSTHus1 protein used for a functional assay that could be taken as a basis for our further experiments (Cai *et al.*, 2000). Contrary to Rad1 and Rad9, Hus1 does not have such different codon frequencies as compared to *E.coli* focussing on the critical codons for its bacterial expression. Therefore, two 'conventional' strains, *E.coli-TB1* and *E.coli-TG1*, were tested in addition to *E.coli BL21-Codon Plus-RIL* paying regard to the influence of a different genetic background on the level of soluble expressed recombinant protein. Considering the differences between the bacterial expression of GSTHus1 protein in these three strains, different expression levels were in fact found, indicating that *E.coli-TB1* is clearly superior in expressing high yields of recombinant GSTHus1 and additionally high amounts of soluble protein. The observation that *E.coli–TB1* shows better yields of soluble GSTHus1 recombinant protein than the special *E.coli BL21*-Codon *Plus-RIL* strain could indicate that codon bias is not the main obstacle to express this checkpoint protein, which corresponds with the fact that the codon frequencies of GSTHus1 are not such different as compared to *E.coli.* For the efficient bacterial expression of soluble Hus1 recombinant protein other parameters seemed to play a critical role, such as different expression conditions or different fusion partners. Considering the time course analysis and the different expression conditions tested for GSTHus1 expressed in *E.coli-TB1* the amount of soluble protein seemed to be increased at a lower cell density at the time of induction (A_{600} =0.5). This supports the thesis that a lower expression level can lead to a higher amount of soluble expressed recombinant protein. Nevertheless, it must be mentioned that even with these optimised conditions, GSTHus1 was expressed mostly insoluble and formed inclusion bodies. After the expression of GSTHus1 in a large-scale culture using the optimised conditions established and the following two steps of purification an amount of 0.043mg/ml soluble and purified GSTHus1 protein was reached which was far below the amount expressed for Rad1 and Rad9 (Table 10). Thereby, some degradation was still present after the second column and appeared to be difficult to separate by further purification steps. Nevertheless, this purified form of recombinant Hus1 protein was shown to be most

likely properly folded by its specific binding to Rad9 *in vitro* and should thereby be suitable for further biochemical or structural studies. It can be assumed that expressing Hus1 as a different fusion protein results in higher amounts of soluble expressed recombinant protein. Therefore, it could be reasonable to test additional fusion partners, primarily MBPHus1 fusion protein, to find out if Hus1 could be bacterially expressed in higher yields and still shows functional activity.

Table 10. Yields of GSTRad1, MBPRad9 and GSTHus1 recombinantly produced in *E.coli* [1]

Protein	**mg/ml [2]**	**ml [2]**	**mg total [2]**	**mg/l culture [3]**
GSTRad1	1.33	1.3	1.73	0.975
MBPRad9	0.997	1.4	1.40	1.21
GSTHus1	0.043	0.75	0.032	0.011

1 the protein amount was quantified by Bradford assay **2** only the main pool was included **3** all pools were included

4.4. Conclusion

During this thesis it became clear that optimised bacterial expression of a recombinant protein depends on numerous parameters that can be individually varied. Therefore the conditions for optimised expression of a specific protein have to be established empirically. According to the experiments performed with Rad9, MBP was far superior to glutathione-S-transferase as a 'solubilizing' partner and showed higher yields of expression. Additionally *E.coli BL21-Codon Plus* strains could in fact improve the expression level of some special recombinant proteins. Thereby it must be checked if codon bias is likely to be a main obstacle for the expression of this protein by calculating its codon frequencies. In other cases, such as Hus1, codon bias does not play that critical role and other *E.coli* strains with different genetic background can be superior in its expression. Therefore, a time-course analysis testing different expression conditions by small-scale cultures seemed to be indispensable for establishing optimised expression conditions for each individual protein. Thereby, good yields of soluble, homogeneous and properly folded protein can be reached by bacterial expression systems.

5. Summary

The human checkpoint proteins Rad9, Rad1 and Hus1 are three DNA quality control proteins that play a critical role in cell cycle checkpoint signalling pathways. These checkpoints are activated due to DNA damage, slow down cell cycle progression and coordinate DNA repair. Failure of this cell cycle surveillance mechanism can cause genomic instability which could eventually lead to cancer formation in mammals.

Soluble and functionally active proteins are required for biochemical and structural analysis of Rad9, Rad1 and Hus1. It is known that these three checkpoint proteins are extraordinarily difficult to express in *E.coli* and only little and incomplete data was collected on their bacterial expression. In this thesis different *E.coli*-strains were used focussing on special *E.coli BL21-Codon Plus* strains, which selectively express certain amino-acids in abundance. Furthermore, different fusion partners and expression conditions were tested for each individual checkpoint protein in order to empirically establish optimised expression conditions. It was shown that soluble and properly folded Rad1 can be expressed in good yields as a GST-tagged protein using the *E.coli BL21-Codon Plus-RIL* strain if optimised expression conditions were established. For Rad9 the best yield of soluble expressed protein was found as a MBP-tagged protein using the *E.coli BL21-Codon Plus-RP* strain. A reasonable amount of soluble recombinant MBPRad9 was purified and shown to be properly folded by its direct and specific interaction with Rad1 and Hus1 *in vitro*. Hus1 was found to be the most insoluble of the three checkpoint proteins analysed. Despite this property, a certain amount of GSTHus1 was bacterially expressed in *E.coli-TB1* in a soluble and properly folded form. Nevertheless, the bacterial expression of Hus1 needs to be further optimised and additional fusion partners need to be tested. In summary, *E.coli BL21-Codon Plus* strains are a suitable system for the production of the two checkpoint proteins Rad1 and Rad9, and to some extent also of Hus1, which can be functionally and biochemically tested.

6. Zusammenfassung

Die drei Checkpoint-Proteine Rad9, Rad1 und Hus1 spielen eine wichtige Rolle bei der Kontrolle der replizierten DNA an Kontrollpunkten (checkpoints) des Zellzyklus. Diese werden als Folge eines DNA-Schadens aktiviert, verlangsamen oder stoppen die DNA-Replikation und koordinieren die DNA-Reparaturmechanismen. Wenn diese Kontrollmechanismen der Zelle versagen, kann dies zur Instabilität des Genoms führen und damit letzten Endes zur Entwicklung von Krebs beitragen.

Für biochemische Untersuchungen und Strukturanalysen von Rad9, Rad1 und Hus1 sind lösliche und funktionell aktive Proteine erforderlich. Es ist jedoch bekannt, dass diese drei Checkpoint-Proteine in *E.coli* ausserordentlich schwierig zu exprimieren sind und bisher nur wenige und unvollständige Daten darüber publiziert wurden. Im Rahmen der vorliegenden Dissertation wurden verschiedene *E.coli* Stämme getestet, wobei hauptsächlich spezielle *E.coli BL21-Codon Plus* Stämme zum Einsatz kamen, welche gewisse Aminosäure-Codons in einem hohen Masse exprimieren. Ausserdem wurden unterschiedliche Fusionspartner und Expressionsbedingungen für die einzelnen Checkpoint-Proteine getestet, um auf empirischem Weg optimale Bedingungen für deren bakterielle Produktion zu etablieren. Es konnte gezeigt werden, dass Rad1 als GST-Fusionsprotein in *E.coli BL21-Codon Plus-RIL* Stämmen bei optimierten Expressionsbedingungen in löslicher und richtig gefalteter Form und in ausreichender Menge exprimiert werden kann. Rad9 konnte als MBP-Fusionsprotein in *E.coli BL21-Codon Plus-RP* Stämmen in löslicher Form und genügender Menge hergestellt werden. Seine korrekte Faltung wurde durch die spezifische und direkte Interaktion mit Rad1 und Hus1 *in vitro* gezeigt. Hus1 scheint das am wenigsten lösliche der drei getesteten Checkpoint-Proteine zu sein. Trotz dieser Eigenschaft konnte eine gewisse Menge an löslichem und richtig gefaltetem Protein als GSTHus1 in *E.coli-TB1* hergestellt werden. Trotzdem muss die bakterielle Expression von Hus1 noch verbessert werden, indem man weitere Fusionspartner untersucht. Zusammenfassend kann ausgesagt werden, dass *E.coli BL21-Codon Plus* Stämme sich eignen, um genügende Mengen der zwei Checkpoint-Proteine Rad1 und Rad9, sowie in beschränktem Ausmass Hus1 zu isolieren, um diese biochemisch und funktionell testen zu können.

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8. Acknowledgements

I would like to sincerely thank the following colleagues for their assistance during this dissertation project:

- Prof. Dr. U. Hübscher for his supervision, guidance and refereeing this thesis.

- Prof. Dr. Hans Lutz for co-refereeing this thesis.

- Dr. K. Dahm for her supervision, guidance and technical assistance during the thesis project.

- Last but not least all my laboratory mates from the Institute of Veterinary Biochemistry and Molecular Biology for their practical and theoretical suggestions and their support.

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